Contribution of Cholesterol and Phospholipids to Inhibitory Effect of Dimethyl-β-Cyclodextrin on Efflux Function of P-Glycoprotein and Multidrug Resistance–Associated Protein 2 in Vinblastine-Resistant Caco-2 Cell Monolayers

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Purpose. The purpose of this study is to reveal the contribution of membrane components to the inhibitory effect of 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CyD) on P-glycoprotein (P-gp) and multidrug resistance–associated protein 2 (MRP2) function in vinblastine-resistant Caco-2 (Caco-2R) cell monolayers.

Methods. The transport of rhodamine-123 and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was studied in Caco-2R cell monolayers. P-gp and MRP2 residing in the monolayers and releasing in cell supernatants were detected by Westerrn blotting. The mRNA levels of *MDR1* and *MRP2* were detected by reverse transcription-polymerase chain reaction (RT-PCR) method. Cholesterol, phospholipids, and proteins were mainly determined by each assay kit.

Results. Of various β-cyclodextrin derivatives (β-CyDs), DM-β-CyD most significantly impaired the efflux function of P-gp and MRP2 without changing cell viability and membrane integrity. The treatment with CyDs did not change the mRNA levels of *MDR1* and *MRP2*. DM-β-CyD lowered cholesterol content and P-gp level in caveolar membranes. In addition, DM-β-CyD released not only cholesterol and phospholipids but also proteins including P-gp and MRP2 from apical membranes of the monolayers.

Conclusions. DM-β-CyD may impair P-gp and MRP2 function in Caco-2R cell monolayers, probably, at least in part, through the release of these transporters from the apical membranes of monolayers, and the exertion of the inhibitory effect of DM-β-CyD may require the extraction of not only cholesterol but also phospholipids from the monolayers.

KEY WORDS: caveolae; cholesterol; cyclodextrins; MRP2; P-glycoprotein; phospholipids.

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ABBREVIATIONS: AP-to-BL, apical to basolateral; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester; BL-to-AP, basolateral to apical; BSA, bovine serum albumin; Caco-2R cells, vinblastine-resistant Caco-2 cells; CyD, cyclodextrin; DM-β-CyD, 2,6-di-*O*-methyl-β-cyclodextrin; DMEM, Dulbecco's modified Eagle's medium; DS, average degree of substitution; HBSS, Hanks' balanced salt solution; HP-β-CyD, 2-hydroxypropyl-β-CyD; M-β-CyD, methyl-β-cyclodextrin; MBS, MES-buffered saline; MDR, multidrug-resistant; MRP2, multidrug resistance–associated protein 2; PBS, phosphate-buffered saline; PE, phycoerythrin; P-gp, P-glycoprotein; PMSF, phenylmethylsulfonylfluoride; SBE-β-CyD, sulfobutylether β-CyD; SDS, sodium dodecyl sulfate.

INTRODUCTION

In apical membranes of intestinal epithelial cells, P-glycoprotein (P-gp) and multidrug resistance–associated proteins (MRP2) are known to express, thus impeding drug absorption, which reduces oral bioavailability (1–4). It has been reported that P-gp exhibits a higher basal ATPase activity in the presence of cholesterol than in the absence of it (5) and localizes in caveolae, flask-shaped invaginations of lipid microdomains enriched in cholesterol and glycosphingolipids, of multidrug-resistant cells (6). However, the relevance of caveolae to P-gp and MRP2 function remains unclear.

Cyclodextrins (CyDs) are host molecules that can include various guest molecules. Methyl-β-cyclodextrin (M-β-CyD) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CyD) are a widely used tool for control of cellular levels of cholesterol through their ability to release cholesterol from the plasma membrane of intact cells (7,8). On the other hand, in the pharmaceutical field, natural CyDs and their hydrophilic CyD derivatives such as methylated, hydroxypropylated, and sulfobutylether derivatives are used for improving the water solubility, dissolution rates, and oral bioavailability of lipophilic drugs (9). Especially, 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CyD) generally possesses the most potent improving effect on oral bioavailability of drugs with extremely poor aqueous solubility (10). In fact, we demonstrated that DM-β-CyD significantly improves oral bioavailability of cyclosporin A and tacrolimus through the solubilizing effect on the hydrophobic drugs (11,12). Recently, we revealed that the improving effect of DM-β-CyD may be associated with the inhibition of P-gp and MRP2 efflux function from the results using Caco-2 cell monolayers, which are a widely used tool to estimate the availability of drugs (13,14), through a release of these transporters as a secondary effect of the extraction of cholesterol from caveolar membranes. However, it is not clear that the inhibitory effect of DM-β-CyD on the efflux activity is involved in only cholesterol-depletion in caveolar membranes. In the current study, therefore, we investigated the inhibitory effects of DM-β-CyD on the function of P-gp and MRP2, and the release of membrane components such as phospholipids, cholesterol, and total proteins were compared with those of other β-CyDs in vinblastine-resistant Caco-2 (Caco-2R) cells, which are feasible for evaluation of the effects of CyDs on the extraction of membrane components because of the high extent of P-gp and cholesterol compared with Caco-2 cells (13).

MATERIALS AND METHODS

Materials

DM-β-CyD and HP-β-CyD with an average degree of substitution (DS) of the hydroxypropyl group of 4.8 were obtained from Nihon Shokuhin Kako (Tokyo, Japan). M-β-CyD (DS = 10.5 to 14.7), indomethacin, and filipin were purchased from Sigma Chemical (St. Louis, MO, USA). Cyclosporin A was a gift from Sandz (Tokyo, Japan). Rhodamine 123 and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester (BCECF-AM) were purchased from Molecular Probes (Eugene, OR, USA). [^{3}H]mannitol was obtained from NEN Life Science Products (Boston, MA, USA). C219 anti-human P-gp and M_2 I-4 anti-

human MRP2 monoclonal antibodies were purchased from Signet Laboratories (Dedham, MA, USA) and Alexis Biochemicals (San Diego, CA, USA), respectively. C13630 anticaveolin polyclonal antibody was obtained from BD Transduction Laboratories (Osaka, Japan). Peroxidase-conjugated sheep anti-mouse IgG and peroxidase-conjugated donkey anti-rabbit IgG antibodies were purchased from Amersham-Pharmacia Biotech (Buckinghamshire, UK). Vinblastine was from Wako Pure Chemical Industries (Osaka, Japan). Deoxyribonuclease I (DNase I) and ribonuclease inhibitor (RNase inhibitor) were from Nippon Gene (Toyama, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Reverse transcriptase (SuperScript II) and Taq polymerase (AmpliTaq Gold) were from GibcoBRL (Gaithersburg, MD, USA) and Applied Biosystems (Tokyo, Japan), respectively. All other chemicals and solvents were of analytical reagent grade.

Cultivation of Caco-2R Cells

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Caco-2R cells were established by continuous exposure of cells to gradually increasing concentrations of vinblastine and were maintained in medium supplemented with vinblastine at 10 nM according to the method of Doppenschmitt *et al.* (15). Caco-2R cells between passage 40 and 60 were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% nonessential amino acids, 2 mM L-glutamine, 0.45% D-glucose, 100 U/ml of penicillin G, and 100 μg/ml of streptomycin.

Cytotoxicity of β-CyDs

Intestinal mitochondrial enzymatic activity was assayed using a Cell Counting Kit (WST-1 method) from Wako Pure Chemical Industries as described previously (16). Briefly, Caco-2R cells were seeded at 5×10^4 cells/well onto 96-well microplates (Iwaki, Tokyo, Japan) and incubated for 1 day in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. Cells were washed three times with Hanks' balanced salt solution (HBSS, pH 7.4) and then incubated for 1 h with 100 μl of HBSS containing CyDs and Tween 20 at various concentrations at 37°C. After three washes with HBSS to remove CyDs and Tween 20, 100 μl of fresh HBSS and 10 μl of WST-1 reagent were added to the plates and incubated for 2 h at 37°C. The absorbance at 450 nm against a reference wavelength of 620 nm was determined with a microplate reader (Model 550, BIO-RAD, Tokyo, Japan). The osmolarity of HBSS (pH 7.4) containing CyDs and Tween 20 was determined with an Osmostat OM-6040 (Arkray Factory, Konan, Japan) by the freezing-point method, and, if necessary, the solutions containing β-CyDs were adjusted to the physiological osmolarity with sodium chloride.

Transepithelial Electrical Resistance

Caco-2R cells were grown on a Transwell of 6-wells and were cultured for 15–21 days before use for the transepithelial electrical resistance (TEER) study. Caco-2R cell monolayers were equilibrated in prewarmed HBSS (37°C, 1.5 ml on the apical side and 2.6 ml on the basolateral side) before starting the experiment. The HBSS on the apical side was replaced with 1.5 ml of prewarmed HBSS in which were dissolved CyDs at various concentrations, and the TEER of the monolayers was measured at indicated time intervals as mentioned above (initial value at time = 0). The TEER of the cell monolayers was calculated according to Eq. (1):

$$\text{TEER} = (R_{\text{total}} - R_{\text{blank}})\ A\ (\text{ohm} \cdot \text{cm}^2) \qquad (1)$$

where R_{total} is the resistance measured, R_{blank} is the resistance of control filters without cells, and A is the surface area of the polycarbonate membranes (4.7 cm^2). The average value of TEER for Caco-2R cell monolayers was 737.9 ± 32 ohm·cm^2, initial values were set to 100%, and the TEER at different points in the presence of CyDs was normalized by dividing by the corresponding control value.

Transport Studies

The effects of treating apical membranes of Caco-2R cell monolayers with CyDs on the transport of [^{3}H]mannitol (a marker of paracellular transport) and the effects of pretreatment of the membranes with CyDs on the transport of rhodamine 123 (a substrate of P-gp) and BCECF (substrate of MRP2) across Caco-2R cell monolayers were studied in transport buffer (10 mM HEPES and 25 mM glucose in HBSS, pH 7.4) at 37°C. Caco-2R cells were grown on a Transwell of 6-wells, and were cultured for 15–21 days before use for the transport study. These monolayers were washed with transport medium, and 1.5 ml and 2.6 ml of transport medium were added to the apical and basolateral sides, respectively. To measure the apical to basolateral (AP-to-BL) and the basolateral to apical (BL-to-AP) transport, the test solution was included in the apical and basolateral sides, respectively. At the designated time, 100 μl of transport buffer from the basolateral or apical side was withdrawn and replaced with an equal volume of transport buffer, respectively. [^{3}H]mannitol (50 nM, 1 μCi/ml) was added to the apical side's transport buffer in the absence and presence of CyDs at various concentrations and incubated for 60 min. On the other hand, the apical membranes were pretreated with 10 mM CyDs for 30 min for the transport studies of rhodamine 123 and BCECF. After washing the apical membranes, rhodamine 123 (5 μM) or BCECF-AM (5 μM) was added to the apical or basolateral side's transport buffer. For other inhibitors, the efflux of rhodamine 123 or BCECF was determined in the presence of 5 μM cyclosporin A or 200 μM indomethacin in the apical and basolateral side's transport buffers without pretreatment. The concentrations of [^{3}H]mannitol in the transport buffer were determined with a liquid scintillation counter (LSC-3500, Aloka, Tokyo, Japan). The concentrations of rhodamine 123 and BCECF in the transport buffer were determined by high performance liquid chromatography (HPLC) and spectrofluorometric method, respectively. The HPLC conditions for rhodamine 123 were as follows: a Hitachi L-6000 pump (Tokyo, Japan) and a Shimadzu RF-550A spectrofluorometric detector (Tokyo, Japan); a Hitachi D-2500 Chromato-Integrator (Tokyo, Japan); a Tosoh TSK gel ODS-80TM column (4.6 × 150 mm, Tokyo, Japan) (detector wavelength: excitation 507 nm, fluorescence 529 nm); a mobile phase of acetonitrile/1% (v/v) acetic acid (2:3 v/v); and a flow rate of 1.0 ml/min. The fluorophotometer for BCECF was a Hitachi F-4500, and excitation and fluorescence wavelengths were 439 nm and 525 nm, respectively. The apparent permeability coefficient (P_{app}) was calculated according to Eq. (2):

$$P_{app} = (dQ/dt)(A \cdot C_0) \quad (2)$$

where dQ/dt is the flux across the monolayer (mol/s), A is the surface area of the membrane (cm^2), and C_0 is the initial drug concentration (mol/ml).

Western Blotting

P-gp, MRP2, and caveolin released from the monolayers were detected by Western blotting. The monolayers were treated with CyDs for 30 min, and the apical side's transport buffer was collected, and then the 20%(v/v) trichloroacetic acid in equal proportion to the buffer was added to precipitate proteins. After being placed on ice for 15 min, the suspension was centrifuged, and then the pellet was washed with 0.5 ml of acetone and dissolved with 20 μl of 1 × sample buffer [2% (w/v) SDS, 10% (w/v) glycerol, 6% (v/v) β-mercaptoethanol in Tris-HCl buffer (pH 6.8)]. The resulting samples were separated by 7% (w/v) and 15% (w/v) SDS-PAGE for the assay of P-gp and MRP2, and caveolin, respectively, and transferred onto PVDF membranes (NEN Life Science Products). The membranes were blocked with 5% (w/v) skim milk in PBS containing 0.1% (w/v) Tween 20 and incubated with C219 anti-human P-gp or M_2I-4 anti-MRP2 monoclonal antibody or C13630 anti-caveolin polyclonal antibody overnight at 4°C. After a wash, the membranes were incubated with peroxidase-conjugated sheep anti-mouse IgG or peroxidase-conjugated donkey anti-rabbit IgG for the assay of P-gp and MRP2, and caveolin, respectively, and were washed three times. Specific bands were detected using the ECL Western blotting analysis kit (Amersham-Pharmacia Biotech) according to the manufacturer's protocol. The bands were detected using the Lumino-image analyzer LAS-1000 plus (Fujifilm, Tokyo, Japan). In some experiments, 10 mM DM-β-CyD–containing transport buffer preloaded with 1.6 mM cholesterol by incubation with free cholesterol for 7 days was used.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction Analysis

The apical membranes of Caco-2R cell monolayers were treated with CyDs for 30 min. Then, the membranes were scraped off by treatment with 0.05% (w/v) trypsin/0.02% (w/v) EDTA as described previously (19). The cells were lysed, and total RNA was extracted using a RNeasy Mini Kit (Qiagen, Tokyo, Japan). Next, the samples were treated with DNase I (1 U) and RNase inhibitors (35 U) for 30 min at 37°C, and complementary DNA was synthesized by reverse transcription using the human *MDR1* gene reverse primer (5′-TTCTGGATGGTGGACAGGCGGTGA-3′), *MRP2* gene reverse primer (5′-ACGAAACCAAAGGCACTCCAGA-3′), or β-*actin* gene reverse primer (5′-GAAGCATTTGCGGTGGACGAT-3′) and reverse transcriptase (SuperScript II). Approximately, 0.1 μM human *MDR1*, *MRP2,* or β-*actin* gene reverse primer was annealed to 3 μg of total RNA and extended with reverse transcriptase (200 U) in a buffer containing 4 μl of 5 × first strand buffer, 10 mM dithiothreitol, and 0.5 mM deoxynucleotide triphosphates for 50 min at 42°C. Amplification of polymerase chain reaction (PCR) was carried out in a Takara PCR Thermal Cycler (Tokyo, Japan). PCR was conducted in a total volume of 100 μl with 2 μl of cDNA, 0.5 μM primers, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, and 2.5 U of Taq DNA polymerase (AmpliTaq Gold). The forward primers for the human *MDR1*, *MRP2,* and β-*actin* gene were 5′-GAGGTGAAGAAGGGCCAGACG-3′, 5′-GAAGACGATGACTATGGGCTGA-3′, and 5′-TCCTGTGGCATCCACGAAACT-3′, respectively. PCR was performed for 25 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 120 s. We confirmed that these amplifications did not reach saturation under these experimental conditions. The amplified products were analyzed on 2% (w/v) low melting temperature agarose gels containing 0.1 μg/ml of ethidium bromide.

Caveolar Membranes Isolation

Low-density caveolar membranes-enriched domains were isolated by a carbonate-based fractionation method as described previously (17). In brief, Caco-2R cell monolayers cultured in 150-mm dishes were scraped into 2 ml of 0.5 M sodium carbonate (pH 11) and homogenized extensively using a Potter-elvehjem type homogenizer (10 strokes), a Polytron tissue grinder (three 10-s bursts at medium speed), and a sonicator (three 20-s bursts). The resulting homogenate was brought to 45% (w/v) sucrose by the addition of 2 ml of 90% (w/v) sucrose in MES-buffered saline (MBS; 25 mM MES, pH 6.5, 150 mM NaCl) and overlaid with two layers of 35% (w/v) and 5% (w/v) sucrose in MBS containing 0.25 M carbonate (4 ml each). The gradient was then centrifuged at 200,000 × *g* for 18 h using a Beckman SW41Ti rotor (Palo Alto, CA, USA). For the analysis of the resulting gradient, 12 fractions (each 1 ml) were collected from the top to the bottom of the gradient. Ten microliters of each fraction were subjected to SDS-PAGE for the detection of P-gp, MRP2, and caveolin, respectively. To compare the levels of P-gp, MRP2, and caveolin inside and outside the caveolar membranes, samples from caveolar membranes and non-caveolar membranes fractions which are equivalent to 4 μg and 10 μg total proteins, respectively, were loaded in each lane, and then electrophoresis and Western blotting were performed as described above. The band density was quantitatively determined by NIH image. On the other hand, the cholesterol mass of each fraction obtained from the sucrose gradient was determined using a Cholesterol Kit Wako (Wako Pure Chemical Industries).

Efflux of Cholesterol, Phospholipids, and Total Proteins

The Caco-2R cell monolayers were incubated for 24 h in growth medium supplemented with 10% FBS containing [^{3}H]cholesterol (5 μCi/ml). Prior to experiments, the cell monolayers were washed three times with transport buffer and incubated in test solutions for 30 min. The concentrations of [^{3}H]cholesterol in test solutions were determined with a liquid scintillation counter as described above. The concentrations of phospholipids and total proteins released from the monolayers in the apical transport buffer were determined using a Phospholipids Kit Wako (Wako Pure Chemical Industries) and a BCA protein assay kit (Pierce, Rockford, IL, USA), respectively.

Statistical Analysis

Data are given as the mean ± SEM. Statistical significance of means for the studies was determined by analysis of

variance followed by Scheffe's test. p values for significance were set at 0.05.

RESULTS

Effects of β-CyDs on Cytotoxicity and Integrity of Caco-2R Cell Monolayers

The cytotoxicity of β-CyDs toward Caco-2R cells was studied using the WST-1 method (Fig. 1). Tween 20, a nonionic surfactant, was used as a positive control and eventually provided almost complete cytotoxicity even at 8.9 mM. HP-β-CyD and SBE7-β-CyD were not cytotoxic to Caco-2R cells up to 100 mM. On the other hand, DM-β-CyD and M-β-CyD reduced the viability, and the cytotoxic effect of DM-β-CyD was higher than that of M-β-CyD.

To evaluate the integrity of tight junctions of Caco-2R cell monolayers as a molecular barrier, the effects of β-CyDs on the TEER and the ability of [^{3}H]mannitol to permeate the monolayers were studied. As shown in Table I, 100 mM HP-β-CyD and SBE7-β-CyD did not lower the TEER value, whereas 30 mM DM-β-CyD and 50 mM M-β-CyD reduced the TEER value. The permeation of [^{3}H]mannitol was significantly increased by treatment with DM-β-CyD and M-β-CyD at the concentrations of 30 mM and 50 mM, respectively, but 100 mM HP-β-CyD and SBE7-β-CyD did not (Table I). Taken together, these results suggest that methylated β-CyDs strongly interact with Caco-2R cell monolayers at higher concentrations.

DM-β-CyD Inhibits P-gp and MRP2 Function

To elucidate whether β-CyDs inhibit the P-gp- and MRP2-mediated substrate efflux from Caco-2R cell monolayers, the effects of various β-CyDs on apical to basolateral (AP-to-BL) and basolateral to apical (BL-to-AP) transports of rhodamine 123 and BCECF, representative P-gp and MRP2 substrates, respectively, were examined. In fact, BCECF-AM, a lipophilic derivative of BCECF, a substrate of MRP2, was used, and we confirmed that BCECF-AM or BCECF is not a substrate of P-gp (data not shown). In order to evaluate correctly the effects of β-CyDs on the P-gp and MRP2 function, Caco-2R cell monolayers were pretreated with 10 mM β-CyDs for 30 min, as β-CyDs provided neither cytotoxicity nor nonspecific paracellular transport under the current experimental conditions (Fig. 1 and Table I), and then the pretreatment can avoid direct interaction between CyDs and the substrates. Figures 2A and 2B show the effects of pretreatment with β-CyDs (10 mM) for 30 min on the BL-to-AP and AP-to-BL permeation of rhodamine 123 through Caco-2R cell monolayers, respectively. When cyclosporin A, a typical P-gp inhibitor, was added to both the apical and the basolateral side transport buffers, the BL-to-AP permeation of rhodamine 123 in the monolayers was markedly decreased, but AP-to-BL permeation of rhodamine 123 was not increased (Figs. 2A and 2B). The lack of enhancing effect of cyclosporin A on AP-to-BL permeation of rhodamine 123 may be due to P-gp–independent AP-to-BL permeation of rhodamine 123 in Caco-2R cell monolayers (18). Likewise, when indomethacin, a typical MRP inhibitor, was added to both the apical and the basolateral sides' transport buffers, BL-to-AP permeation of BCECF was decreased, but AP-to-BL permeation was suppressed as well (Figs. 2B and 2C). The inhibition of both BL-to AP and AP-to-BL permeation of BCECF could be attributed to the nonspecific inhibitory effect of indomethacin to both MRP2 and MRP3 (19), as the extent of BCECF accumulated in the monolayers in the presence of indomethacin indeed increased approximately 3.9-fold than that in the absence of indomethacin under the current experimental conditions. Anyway, these data indicate that P-gp and MRP2 work well in the monolayers under the current experimental conditions, as these inhibitors markedly suppressed BL-to-AP transport of each substrate. As shown in Figs. 2A and 2B, of the four β-CyDs, only DM-β-CyD inhibited the BL-to-AP transport and augmented the AP-to-

Table I. Effect of β-CyDs Treatment on TEER and Permeation of [^{3}H]Mannitol through Caco-2R Cell Monolayers

System	TEER (% of initial value)	Papp[a] × 10^7 (cm/s)
Control	103.6 ± 0.6	2.31 ± 0.4
With DM-β-CyD		
(10 mM)	101.9 ± 1.0	2.58 ± 0.6
(15 mM)	100.5 ± 1.6	4.02 ± 0.7
(30 mM)	78.4 ± 1.4*	9.6 ± 2.1*
With M-β-CyD		
(15 mM)	105.7 ± 1.3	2.12 ± 0.7
(50 mM)	81.4 ± 1.5*	8.92 ± 1.8*
With HP-β-CyD		
(100 mM)	124.7 ± 1.2*	2.64 ± 0.1
With SBE7-β-CyD		
(100 mM)	99.7 ± 0.5	3.36 ± 0.3

[a] Apparent permeability coefficient of apical to basolateral [^{3}H]mannitol permeation.
The concentration of [^{3}H]mannitol was 50 nM.
* $p < 0.05$ vs. Control.

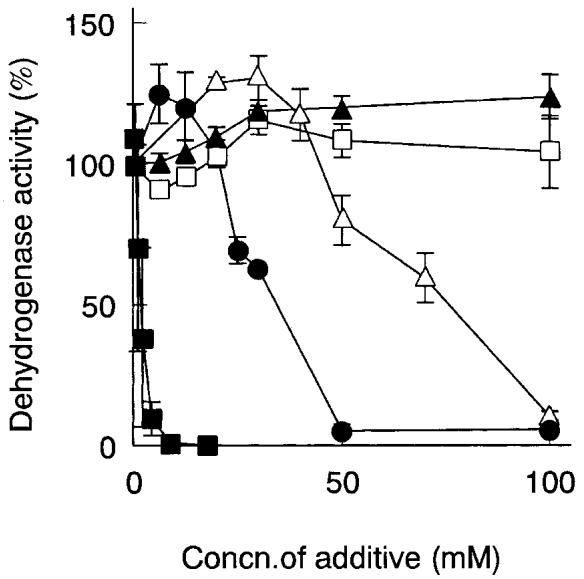

Fig. 1. Cytotoxicity of β-CyDs and Tween 20 in Caco-2R cells in HBSS (pH 7.4) at 37°C: ●, DM-β-CyD; △, M-β-CyD; ▲, HP-β-CyD; □, SBE7-β-CyD; ■, Tween 20. Cells were washed three times with HBSS (pH 7.4) and then incubated for 60 min with 100 μl of HBSS containing CyDs and Tween 20 at various concentrations at 37°C. After three washes with HBSS to remove CyDs and Tween 20, cell viability was assayed by the WST-1 method. Each point represents the mean ± SEM of 6 experiments.

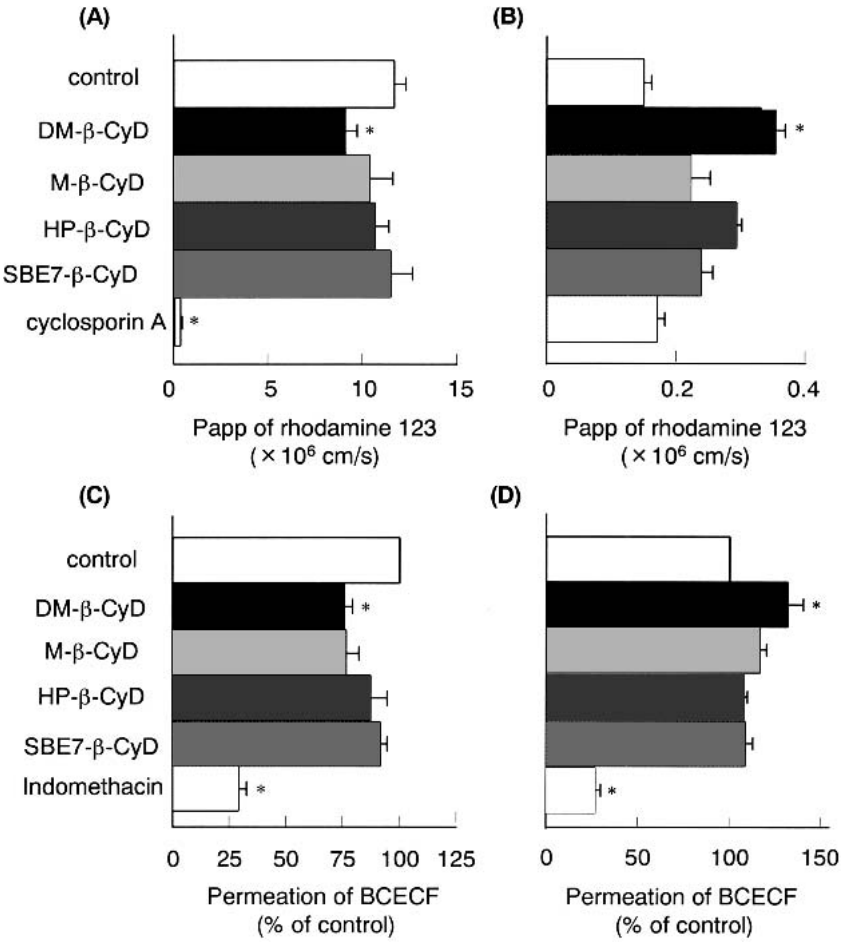

Fig. 2. Inhibitory effects of various agents on (A, C) basolateral to apical and (B, D) apical to basolateral permeation of (A, B) rhodamine 123 and (C, D) BCECF through Caco-2R cell monolayers. The apical membranes of Caco-2R cell monolayers were pretreated with β-CyDs at 10 mM for 30 min at 37°C. After a wash of the apical membranes, (A, B) 5 μM rhodamine 123 or (C, D) 5 μM BCECF-AM was added to the apical or the basolateral side's transport buffer. The concentrations of rhodamine 123 and BCECF were determined by HPLC and a fluorescence spectrophotometer, respectively. Each value represents the mean ± SEM of 3–7 experiments. *$p < 0.05$ vs. rhodamine 123 alone and BCECF alone, respectively.

BL transport of rhodamine 123 compared to the control. Figures 2C and 2D show the effects of β-CyDs on MRP2 function in Caco-2R cell monolayers. In the monolayers, BL-to-AP efflux and AP-to-BL influx were inhibited and promoted by pretreatment with DM-β-CyD, respectively (Figs. 2C and 2D). M-β-CyD inhibited the BL-to-AP efflux to the same extent as DM-β-CyD, but the inhibitory effect of M-β-CyD was statistically insignificant and other β-CyDs changed neither the efflux nor influx of BCECF. These results indicate that of the four β-CyDs used here, only DM-β-CyD significantly inhibits the P-gp- and MRP2-mediated efflux from Caco-2R cell monolayers.

DM-β-CyD Releases P-gp and MRP2 from Caco-2R Cell Monolayers

Next, we investigated the inhibitory mechanism of DM-β-CyD on P-gp and MRP2 function in Caco-2R cell monolayers. It is conceivable that the inhibitory mechanism of DM-β-CyD totally differs from that of the competitive inhibitors to P-gp and MRP2 such as cyclosporin A and indomethacin because CyDs are unable to enter in cells (10). In addition, we previously reported that DM-β-CyD releases P-gp and MRP2 from Caco-2 cell monolayers into the apical side's transport buffer (14). Thus, we examined whether β-CyDs allow the release of P-gp and MRP2 from Caco-2R cell monolayers. First of all, we confirmed that P-gp and caveolin overexpress in Caco-2R cells compared to Caco-2 cells (Fig. 3A). Bands corresponding to P-gp and MRP2 were clearly observed in the apical side's transport buffer after treatment with DM-β-CyD, but bands were observed after that with neither M-β-CyD nor HP-β-CyD (Fig. 3B). On the other hand, the addition of free cholesterol to DM-β-CyD–containing buffer completely inhibited the release of P-gp and MRP2 into the apical side's transport buffer (Fig. 3C), suggesting that the ability of inclusion complexation of DM-β-CyD is involved in the release of these transporters.

P-gp is known to localize in caveolae of multidrug-resistant cells (6), but MRP2 remains unknown. To examine whether P-gp and MRP2 localize in caveolae of the monolay-

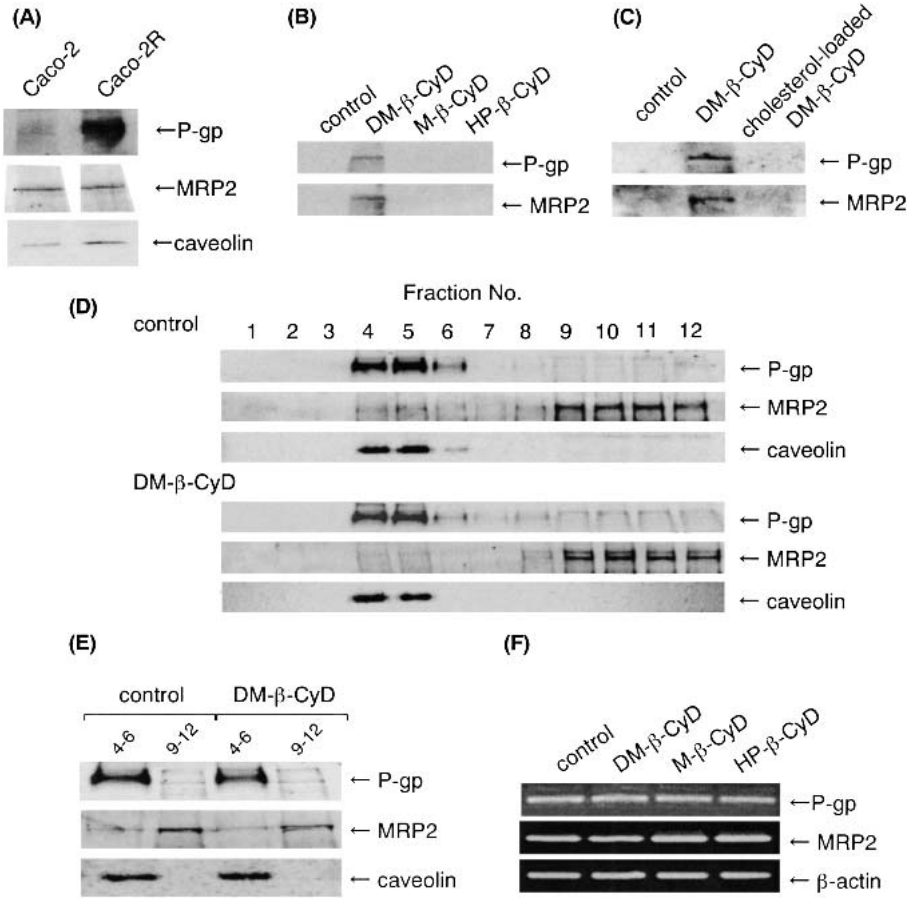

Fig. 3. The releasing effect of β-CyDs on P-gp and MRP2 from the Caco-2R cell monolayers and their caveolae. (A) The comparison of various proteins' expression in-between Caco-2 and Caco-2R cell monolayers. (B) The monolayers were treated with 10 mM β-CyDs for 30 min at 37°C, and then P-gp and MRP2 levels released in the apical side's transport buffer were assayed by immunoblotting. (C) Effects of an addition of cholesterol to DM-β-CyD–containing apical side's transport buffer on the release of P-gp and MRP2 in the buffer of Caco-2R cell monolayers. (D) Effect of DM-β-CyD on P-gp and MRP2 expression in caveolae-enriched domains in Caco-2R cell monolayers. The monolayers were treated with 10 mM CyDs for 30 min at 37°C, caveolae were isolated, and the activity of P-gp and MRP2 was assayed by immunoblotting. (E) Comparison of P-gp, MRP2, and caveolin levels in caveolae and non-caveolae fractions of Caco-2R cell monolayers. The monolayers were treated with 10 mM CyDs for 30 min at 37°C, and then P-gp and MRP2 levels in caveolae and non-caveolae domains were assayed by immunoblotting after adjusting the total protein content (4 and 10 μg/lane in caveolae and non-caveolae, respectively). (F) *MDR1* and *MRP2* mRNA levels in Caco-2R cell monolayers. The monolayers were treated with 10 mM CyDs for 30 min at 37°C, and then *MDR1* and *MRP2* mRNA levels in the cells were assayed by RT-PCR.

ers, we isolated caveolar membranes from Caco-2R cell monolayers. As shown in Fig. 3D, P-gp was co-localized with caveolin in the low-density fractions, whereas MRP2 resided in both low- and high-density fractions, suggesting that P-gp localized in caveolae and MRP2 resided in both caveolae and non-caveolae fractions of Caco-2R cell monolayers. Intriguingly, the localization of these proteins was changed by treatment with DM-β-CyD only very slightly, as their band patterns were not changed by the treatment with DM-β-CyD (Fig. 3D). To determine the effect of DM-β-CyD on the expression levels of P-gp, MRP2, and caveolin inside and outside of caveolae factions, samples were loaded in each lane after adjusting the protein content, followed by SDS-PAGE and Western blotting. These results are shown in Fig. 3E. The band density was then determined using NIH image. The treatment of the apical membranes of Caco-2R cell monolayers with DM-β-CyD decreased P-gp and caveolin levels in caveolae to 85 ± 4% and 98 ± 4% (mean ± SEM of 3 experiments), whereas it decreased MRP2 levels to 94 ± 3% and 97 ± 3% (mean ± SEM of 3 experiments) inside and outside of caveolae, respectively. These results suggest that DM-β-CyD attenuated the P-gp level in caveolar membranes and the MRP2 level in both caveolar and noncaveolar membranes, although the decreasing extent was considerably slight (Fig. 3E). However, DM-β-CyD did not lower the caveolin level, suggesting that it failed to disrupt the caveolar structure of Caco-2R cell monolayers. On the other hand, we reported that the treatment with DM-β-CyD did not change the transcription of *MDR1* in Caco-2R cell monolayers (13). Under the same experimental conditions, M-β-CyD or HP-β-CyD

did not change the band densities corresponding to *MDR1* and *MRP2* mRNA in Caco-2R cell monolayers (Fig. 3F). Taken together, these results indicate that the inhibitory effect of DM-β-CyD on P-gp and MRP2 function could be, at least in part, attributed to the release of P-gp and MRP2 from the apical membranes of Caco-2R cell monolayers.

DM-β-CyD Releases Cholesterol, Phospholipids, and Proteins

CyDs are known to induce hemolysis by releasing membrane constituents such as cholesterol and phospholipids (20). Thus, we examined the release of cholesterol, phospholipids, and proteins from Caco-2R cell monolayers. As shown in Figs. 4A–4C, DM-β-CyD released most significantly [^{3}H]cholesterol, phospholipids, and total proteins, and then M-β-CyD released [^{3}H]cholesterol more than HP-β-CyD and SBE7-β-CyD. Therefore, these data indicate that of β-CyDs used here, the ability of DM-β-CyD to release membrane lipids and proteins was strongest.

To confirm whether cholesterol is rich in caveolar membranes of Caco-2R cell monolayers, we next determined the cholesterol level inside and outside of caveolar membranes of Caco-2R cell monolayers. As shown in Fig. 4D, cholesterol was observed in low-density fractions (fractions 4–6), consistent with the localization of caveolin. In addition, treatment with DM-β-CyD and M-β-CyD reduced the cholesterol content in caveolar membranes to approximately 60% and 75% of control, respectively (Fig. 4E). These results suggest that the effect of DM-β-CyD to release cholesterol and phospholipids from Caco-2R cell monolayers causes the release of proteins including P-gp and MRP2 from caveolar membranes, resulting in lowering of P-gp and MRP2 function. To clarify whether P-gp function is changed by the suppression of caveolar membranes function, we next examined the effect of filipin, a cholesterol-binding agent, on BL-to-AP permeation of rhodamine 123 in Caco-2R cell monolayers. As shown in Fig. 4F, it is apparent that filipin suppressed the permeation of rhodamine 123. These results suggest that caveolae are involved in P-gp function in Caco-2R cell monolayers.

DISCUSSION

We demonstrated here that DM-β-CyD markedly inhibits its P-gp and MRP2 function relative to other β-CyDs, probably resulting from the efficient release of the efflux transporters through the extraction of cholesterol as well as phospholipids from the apical membranes.

Of the β-CyDs used here, DM-β-CyD provided cytotoxicity and change in the Caco-2R cell monolayer's integrity at higher concentrations (>25 mM, Fig. 1 and Table I). This may be due to strong interaction of DM-β-CyD with biomembranes, because DM-β-CyD possesses the strongest hemolytic activity among β-CyDs used here (20). With respect to the property of the inclusion complexation of CyDs toward biomembrane components, α-CyD and β-CyD are known to fit phospholipids and cholesterol, respectively (10). In fact, α-CyD and β-CyD include aliphatic moiety and five-number ring of prostaglandin (21), respectively. Thus, we expected that DM-β-CyD and M-β-CyD preferentially extract cholesterol rather than phospholipids from Caco-2R cell monolayers. As expected, it is evident that M-β-CyD released cholesterol from the monolayers. However, DM-β-CyD released cholesterol as well as phospholipids. This extraction ability of DM-β-CyD may result in the strongest interaction with Caco-2R cell monolayers (Fig. 1 and Table I). This different extraction ability between DM-β-CyD and M-β-CyD could be attributed to the slight difference in DS values of methyl group.

The interaction mode of β-CyDs with Caco-2R cell monolayers may be different from that with Caco-2 cell monolayers. Actually, the expression of P-gp and caveolin is confirmed to be higher in Caco-2R cells than in Caco-2 cells (Fig. 3A) (13,14), reflecting our results which the ratio of P_{app} value of BL-to-AP to AP-to-BL permeation of rhodamine 123 showed 77.5 and 33.5 in Caco-2R and Caco-2 cell monolayers, respectively (14). In addition, multidrug-resistant cells have been reported to elicit the different extent of membrane lipids from tumor cells i.e., cholesterol and glycosylceramide levels augment, whereas the lactosylceramide level decreases in multidrug-resistant cells (22,23). Therefore, the expression of proteins and lipids in multidrug-resistant cells definitely differs from parent tumor cells. On the other hand, the interaction of β-CyD with liposomal membranes augmented as cholesterol content in the membranes increased (24). Taken together, the different extent and composition of membrane lipids may lead to the different interaction with various β-CyDs. Thus, we compared the ability of DM-β-CyD to release membrane lipids from Caco-2R and Caco-2 cell monolayers. As a result, the concentrations of phospholipids in cell supernatants after treatment with DM-β-CyD for 30 min in Caco-2R and Caco-2 cell monolayers were 9.2 ± 0.5 μg/ml and 7.1 ± 0.6 μg/ml, respectively, when the same number of cells (4×10^5 cells/dish) were seeded. Likewise, those of total proteins in Caco-2R and Caco-2 cell monolayers were 18.1 ± 1.7 μg/ml and 14.1 ± 1.3 μg/ml, respectively. In addition, the treatment with DM-β-CyD lowered cholesterol content in caveolar membranes of Caco-2R and Caco-2 cell monolayers by 65.2 ± 7.9% and 80.0 ± 4.6%, respectively. Thus, it is evident that the effects of DM-β-CyD to release membrane components were higher in Caco-2R cell monolayers than Caco-2 cell monolayers. These results may allow us to imagine the possibility that DM-β-CyD preferentially interacts with multidrug-resistant cells relative to tumor cells, leading to multidrug resistance cell-specific cytotoxicity.

We demonstrated that P-gp localizes in caveolae, which are sphingomyelin- and cholesterol-rich membrane microdomains, of Caco-2R cell monolayers (Figs. 3D and 3E). However, the physiological role of P-gp localizing in caveolae remains unclear. It is possible that P-gp in caveolae is involved in transport or translocation of lipids such as sphingomyelin and cholesterol, and it could play a crucial role for cell proliferation, signal transduction, and transcytosis (25).

DM-β-CyD significantly may impair the efflux activity of P-gp and MRP2 through the release of P-gp from caveolar membranes and MRP2 from caveolar and non-caveolar membranes of Caco-2R cell monolayers, respectively (Figs. 2 and 3). In the current study, the unique property of DM-β-CyD to release P-gp and MRP2 from the apical membranes into cell supernatant was observed (Fig. 3B). This result was consistent with the finding that only DM-β-CyD lowered P-gp and MRP2 function in Caco-2R cell monolayers (Fig. 2). In addition, the pivotal extraction ability of DM-β-CyD on P-gp and

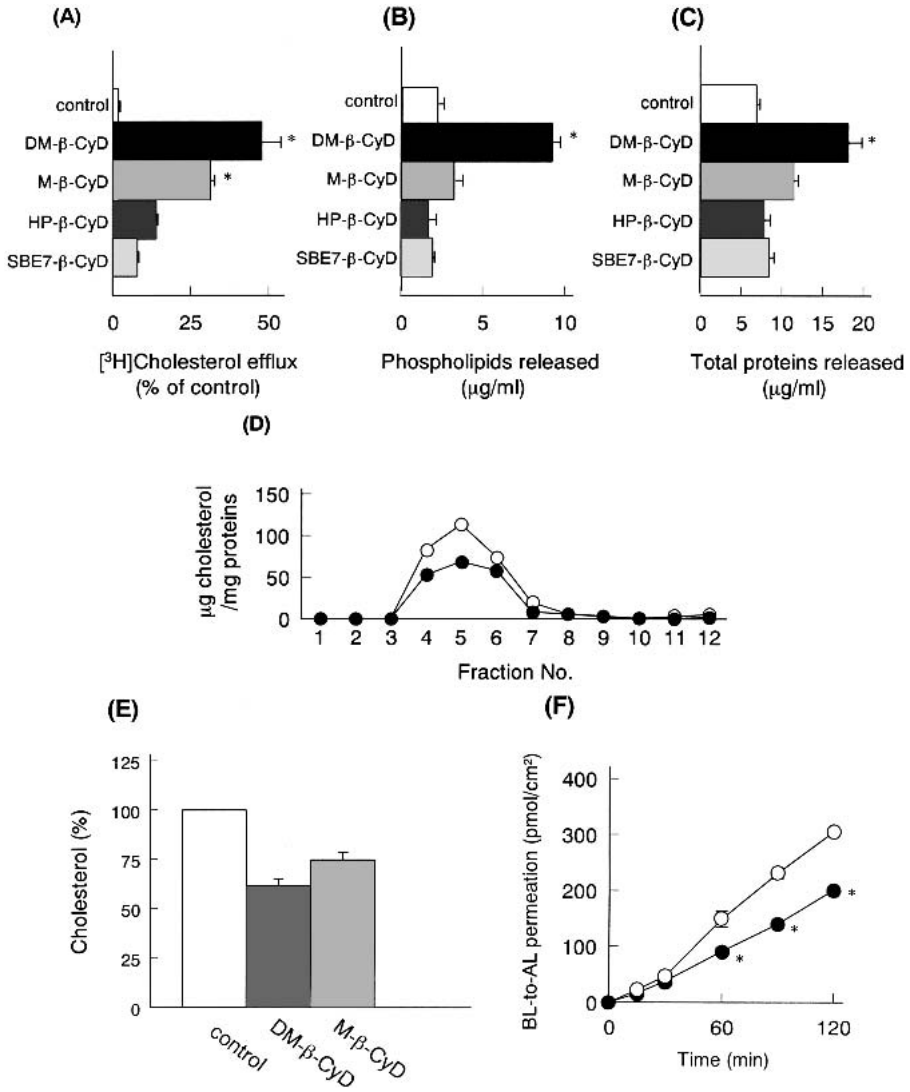

Fig. 4. Effects of β-CyDs on the release of (A) [^{3}H]cholesterol, (B) phospholipids, and (C) total proteins from Caco-2R cell monolayers. The monolayers were labeled with [^{3}H]cholesterol and then treated with 10 mM CyDs for 30 min at 37°C. The radioactivity of [^{3}H]cholesterol in the apical side's transport buffer was assayed using a liquid scintillation counter. The concentrations of phospholipids and total proteins released in apical side's transport buffer were measured using a Phospholipids Kit Wako and BCA protein assay kit. Each value represents the mean ± SEM of 3–11 experiments. (D) Effect of DM-β-CyD on the extent of cholesterol in caveolae and non-caveolae fractions of Caco-2R cell monolayers: ○, control; ●, DM-β-CyD. The monolayers were treated with 10 mM DM-β-CyD for 30 min at 37°C, and the amount of cholesterol in each fraction after sucrose gradient centrifugation was determined with a Cholesterol Kit Wako. The data represent the mean of three experiments. (E) Effect of β-CyDs on the extent of cholesterol retained in caveolae domain of Caco-2R cell monolayers. Bars refer to percent as compared with control, which was defined as 100%. Fractions 4–6 were defined as caveolae fraction. Each value represents the mean ± SEM of 3–5 experiments. (F) Effects of filipin on basolateral to apical permeation of rhodamine 123 through Caco-2R cell monolayers. The apical membranes of Caco-2R cell monolayers were pretreated with filipin for 30 min. After washing, transport studies were performed as described above. ○, control; ●, pretreated with 10 μg/ml of filipin. Each value represents the mean ± SEM of 3 experiments. *$p < 0.05$ vs. control.

MRP2 may also be elicited from the findings that (i) DM-β-CyD did not change the levels of P-gp and MRP2 mRNA (Fig. 3F), (ii) DM-β-CyD failed to translocate P-gp and MRP2 from caveolar membrane domains to non-caveolae, and (iii) DM-β-CyD was unable to translocate or release caveolin from caveolae, suggesting that it did not disrupt caveolar structure of Caco-2R cell monolayers. Taken together, these results may support the crucial ability of DM-β-CyD to release P-gp and MRP2 in the inhibitory effect on their transporter's function.

Next, we examined whether DM-β-CyD releases P-gp and MRP2 through direct interaction. It is well-known that

CyDs interact with proteins weakly as described above (26). For instance, hemolysis induced by CyDs is believed to be exerted by the secondary effect of lipid extraction from the membranes (20). Likewise, the ability of DM-β-CyD to release P-gp and MRP2 from the apical membranes may be due to the inclusion ability because the addition of cholesterol to DM-β-CyD-containing solution failed to release these transporters (Fig. 3C). In addition, we confirmed that almost all of the cholesterols existed in caveolar membranes, and they were released from caveolar membranes by the treatment with methylated β-CyDs (Figs. 4C and 4D). Therefore, it is likely that the effects of DM-β-CyD on P-gp and MRP2 function could be ascribed to the secondary effect on the basis of the release of cholesterol from caveolar membranes of the apical membranes.

Furthermore, we examined whether phospholipids are involved in the ability of DM-β-CyD to release P-gp and MRP2 from Caco-2R cell monolayers. It is certain that DM-β-CyD dominantly released not only cholesterol but also phospholipids relative to other β-CyDs used here. Importantly, M-β-CyD preferentially released cholesterol from the monolayers (Fig. 4), while 2,6-di-*O*-methyl-α-cyclodextrin (DM-α-CyD) selectively released phospholipids from the monolayers (data not shown). However, neither M-β-CyD nor DM-α-CyD released P-gp and MRP2 or inhibited their function in Caco-2R cell monolayers, suggesting that both cholesterol and phospholipids are associated with the ability of DM-β-CyD to release P-gp and MRP2 from the monolayers (data not shown). Therefore, it is possible that DM-β-CyD may inhibit P-gp and MRP2 function through the release of glycosphingolipids from caveolae as well as cholesterol, because glycerophospholipids are known to exist outside of caveolar membranes (27,28). However, the possible involvement in other factors such as membrane fluidization and ATPase activity of these transporters should not be ruled out because the ability of DM-β-CyD to release P-gp and MRP2 was somewhat low.

In the current study, filipin suppressed P-gp function in Caco-2R cell monolayers (Fig. 4F). However, it did not affect MRP2 function (data not shown). These results suggest that the contribution of cholesterol to P-gp function may be different from that to MRP2 function in Caco-2R cell monolayers. In addition, the different role of cholesterol for P-gp and MRP2 function may be, in part, their distinct membrane localization on Caco-2R cell monolayers (Fig. 3D). Thus, the inhibitory effect of filipin may be consistent with the finding regarding the importance of cholesterol in maintenance of P-gp activity and its membrane perturbing function (29). However, the differential effect of M-β-CyD and filipin on B-cell receptor signaling has been reported (30). In fact, DM-β-CyD is unlikely to disrupt caveolae of Caco-2R cell monolayers under the current experimental conditions as described before. Thereby, the detailed mechanism by which what component is most critical for the inhibitory effect of DM-β-CyD on P-gp and MRP2 function in Caco-2R cell monolayers remains unclear. Further work is necessary to understand the elaborate mechanism.

In conclusion, of various β-CyDs used here, DM-β-CyD impaired P-gp and MRP2 function in Caco-2R cell monolayers, at least in part, probably through the release of these transporters from the apical membranes of the cells, resulting from the extraction of both cholesterol and phospholipids from the monolayers. The current results would be useful for understanding the role of membrane lipids in P-gp and MRP2 function in multidrug-resistant cells.

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