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Newly synthetic ceramide-1-phosphate analogs; their uptake, intracellular localization, and roles as an inhibitor of cytosolic phospholipase $A_2\alpha$ and inducer of cell toxicity

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ABSTRACT

Ceramide-1-phosphate (C1P) regulates cellular functions including arachidonic acid (AA) metabolism and modulates cell fate. The mechanism by which C1P is taken up is unclear, and the development of lipophilic analogs may be useful for regulating C1P's actions. We synthesized new mono- and di-methyl-ester (MM and DM, respectively) analogs of C1P with *N*-acyl chains of different lengths, and examined their effects on AA release and cell toxicity. Short-*N*-acyl-DM-C1P analogs including C5- and C6-DM-C1P, but not long-*N*-acyl-DM-C1P analogs, inhibited the release of AA mediated by α type cytosolic phospholipase A_2 ($cPLA_2\alpha$) in Chinese hamster ovary (CHO) cells and the enzymatic activity. Short-*N*-acyl-DM-C1P analogs including C6-DM-C1P caused morphological changes with cell toxicity 24 h after the treatment in three cells lines (CHO, L929, and RLC-18 cells), although the role of AA in the toxicity was not clear. Neither long-*N*-acyl-DM-C1P analogs nor MM-C1P analogs including C6-MM-C1P affected $cPLA_2\alpha$ activity and cell toxicity. Similar to C6-ceramide having a 4-nitrobenzo-2-oxa-1,3-diazole (NBD) group on a C6-*N*-acyl chain (NBD-C6-ceramide), NBD-C6-DM-C1P and C6-DM-C1P-NBD (with a C6-*N*-acyl chain and an NBD-labeled C14-alkyl chain) were accumulated in the Golgi complex, although less C6-DM-C1P-NBD than NBD-C6-DM-C1P was taken up. NBD-C6-ceramide was converted to various metabolites including NBD-C6-sphingomyelin, but both NBD-C6-DM-C1P and C6-DM-C1P-NBD were stable in cells within 2 h. The short-*N*-acyl-DM-C1P analogs acted directly as an inhibitor of $cPLA_2\alpha$ and an inducer of cell toxicity, and may be useful for the regulation of ceramide/C1P-regulated responses.

1. Introduction

Ceramide-1-phosphate (C1P) is emerging as a biological and/or signaling molecule capable of regulating various cellular functions including cell proliferation, apoptosis, phagocytosis, and inflammation [1–5]. There are several reports showing intracellular targets of C1P; direct inhibition of acid sphingomyelinase and direct activation of α type cytosolic phospholipase A_2 ($cPLA_2\alpha$, Refs. [6–8]). While there is a growing list of cellular responses attributed to C1P, it is still unknown how exogenously added C1P signals to produce intracellular effects, and the specific transport systems for C1P uptake have not been identified. It was reported that using organic solvents including dodecane to deliver C1P caused cellular responses with loss of cell viability in a non-specific manner [9]. In contrast, many reports showed that treatment with C1P with and without organic solvents caused cellular responses without cell toxicity [4,10,11]. Although C1P was recently proposed to interact with a putative receptor that is coupled to G proteins in macrophages [12], many cellular effects including the regulation of $cPLA_2\alpha$ appeared to be dependent on intracellular C1P via its unknown uptake system. In cells, C1P is formed from ceramide by ceramide kinase at cellular compartments including the Golgi complex, and readily transported along the secretory pathways to other sites including the plasma membrane [13–15]. C1P is dephosphorylated to form ceramide by lipid phosphate phosphatases in cells [4,9]. The procedure used for C1P's delivery and translocation, as well as the formation of ceramide, may impact the results. At low concentrations, C1P enhanced the survival of cells, while at high concentrations, it caused

Abbreviations: C1P, ceramide-1-phosphate; PLA_2, phospholipase A_2; $cPLA_2\alpha$, α type cytosolic phospholipase A_2; MM- and DM-C1P, mono- and di-methyl-ester analogs of C1P; CHO, Chinese hamster ovary; PAF, platelet-activating factor; PMA, 4β-phorbol 12-myristate 13-acetate; NBD, 7-nitrobenz-2-oxa-1,3-diazole; PAPC, 1-palmitoyl-2-arachidonyl phosphatidylcholine; LDH, lactate dehydrogenase; S1P, sphingosine-1-phosphate.

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cell toxicity with a correlation to the amount of pro-apoptotic ceramide formed [16]. Thus, the development of highly lipophilic and/or cell-permeable C1P analogs that are resistant to metabolism appears to be useful for the regulation of cellular responses such as the release of AA and cell fate. Recently, an analog of C1P (PCERA-1, phospho-ceramide analogue-1) was reported to suppress and increase production of pro- and anti-inflammatory cytokines, respectively, and to be a putative drug for the treatment of inflammatory diseases [17,18].

C1Ps having a short-*N*-acyl chain have been utilized to examine cellular effects as their solubility allows for easy delivery to cells, and C2-C1P and C8-C1P caused similar responses to C1P having a long-*N*-acyl chain [4,19], although they do not always cause the same effects [4,6,10]. We reported that treatment of cells with C2-C1P, which alone had no effect, was an independent activator of the release of AA mediated by cPLA$_2\alpha$ in the presence of Ca^{2+} signaling [20]. By contrast, several reports showed chain length specificity of C1P for activity. C2-C1P could not regulate directly acid sphingomyelinase [6] or cPLA$_2\alpha$ [10], although C1P having an *N*-acyl chain longer than 6 carbons cloud. The role of the *N*-acyl chain length specificity of C1P in the cellular responses is frequently discussed. In the present study, we newly synthesized several mono- and di-methyl-ester analogs of C1P (MM-C1P and DM-C1P, respectively) having *N*-acyl chains of various lengths, and examined their effects on the release of AA and intracellular uptake and distribution in Chinese hamster ovary (CHO) cells. In addition, cell toxicity induced by C1P analogs was examined in three different cell types, CHO cells, L929 mouse fibrosarcoma cells, and RLC-18 rat liver epithelial cells.

2. Experimental procedures

2.1. Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (7.92 TBq/mmol) was from Amersham (Buckinghamshire, UK). Secretory PLA$_2$ (type III from bee venom, No 60500) and platelet-activating factor (PAF) were from Cayman (Ann Arbor, MI). 4β-Phorbol 12-myristate 13-acetate (PMA), A23187, and C1P from bovine brain (C4832) were from Sigma (St. Louis, MO). C2-C1P and C8-C1P were from Biomol (Plymouth Meeting, PA). The methyl-ester analogs of C1P with and without a fluorescent group, 4-nitrobenzo-2-oxa-1,3-diazole (NBD), were synthesized by standard methods in our laboratories and details of the synthesis will be reported elsewhere. The formula, structural abbreviation, and compound number of each C1P analog are shown in Fig. 1 and Table 1. The log *P* values of the analogs were calculated by the CLOGP program (Bio-Loom for Windows, ver. 1.0, BioByte) as parameters of lipophilicity. DM-C1P analogs were dissolved in dimethyl sulfoxide (Wako, Osaka, Japan) prior to dilution in the buffer, and the final concentration of dimethyl sulfoxide was under 0.5%. Other organic solvents such as dodecane, chloroform, and methanol were not used in the present study. MM-C1P analogs were synthesized as ammonium salts, and dissolved in distilled water. The pH of the buffers containing the DM-C1P and MM-C1P analogs was adjusted to 7.4–7.5. The effects of the C1P analogs were usually examined at 10 μM, and the dose-dependency of the analogs was examined in some experiments. The vehicles had no effect on the cellular responses including the release of AA for 1–3 h, cell morphology, and/or fate 24 h after treatment.

2.2. Cell culture

CHO-W11A cells (a stable line of CHO cells expressing PAF receptors at high levels, Ref. [21]) were grown in Ham's F12 medium supplemented with 10% heat-inactivated fetal bovine serum (Thermo Trace, Ltd., Nobel Park, Australia). L929 cells (a murine fibrosarcoma cell line) and RLC-18 cells (a rat liver epithelial cell line, a non-tumor cell line) were cultured in Dulbecco's modified Eagle's medium supplemented with the serum, according to instructions (RIKEN BioResource Center, Saitama, Japan).

2.3. Assay for release of [^{3}H]AA from cells

The release of [^{3}H]AA was determined as described previously [7,11,20]. Briefly, CHO-W11A cells ($(2–3) \times 10^4$) were seeded on

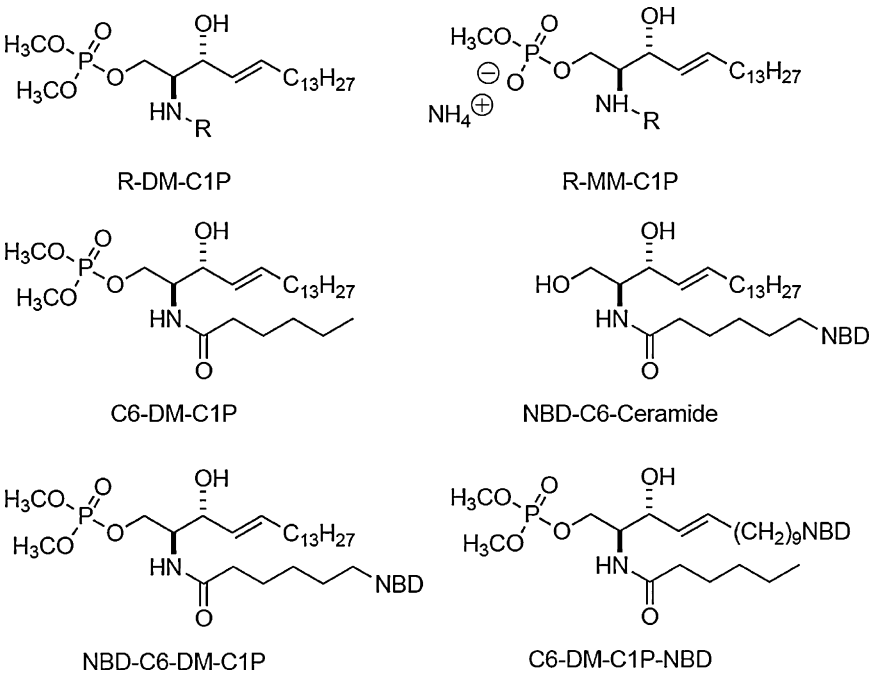

Fig. 1. Formula and structural abbreviation of each C1P analog. [DM] and [MM] show the di- and mono-methylation of a phosphate group, respectively. Full formula of the analog is shown in Supplementary Fig. 1.

Table 1
Newly synthesized methyl-ester analogs of C1P, and their effects on cell morphological changes and/or toxicity.

Reagents (Compound **No**; MW; *C*log*P*)	Cell toxicity		
	CHO cells	L929 cells	RLC-18 cells
Vehicle	No	No	No
C2-C1P	No	No	N.D.
C8-C1P	No	No	N.D.
C2-DM-C1P (**1**; 449.5; 5.11)	Weak	Moderate	Weak
C5-DM-C1P (**2**; 491.6; 6.60)	Strong	Strong	Strong
C6-DM-C1P (**3**; 505.6; 7.23)	Strong	Strong	Strong
C8-DM-C1P (**4**; 533.7; 8.28)	No	No	No
C12-DM-C1P (**5**; 589.8; 10.4)	No	No	No
C16-DM-C1P (**6**; 645.9; 12.5)	No	No	No
C18-DM-C1P (**7**; 674.0; 13.5)	No	No	No
C2-MM-C1P (**8**; 435.5; 5.45)	No	No	No
C5-MM-C1P (**9**; 477.6; 7.04)	No	No	No
C6-MM-C1P (**10**; 491.6; 7.57)	No	No	No
C8-MM-C1P (**11**; 519.7; 8.62)	No	No	No
C12-MM-C1P (**12**; 575.8; 10.7)	No	No	No
C16-MM-C1P (**13**; 631.9; 12.8)	No	No	No
NBD-C6-ceramide	Strong	Moderate	Moderate
NBD-C6-DM-C1P (**14**; 683.7; 8.09)	Strong	Strong	Strong
C2-DM-C1P-NBD (**15**; 571.5; 3.86)	Weak	Weak	Weak
C6-DM-C1P-NBD (**16**; 627.6; 5.98)	Weak	Strong	Strong
C16-DM-C1P-NBD (**17**; 767.9; 11.2)	No	No	No

[DM] and [MM] indicate a di-methylated and mono-methylated phosphate group, respectively. MW, molecular weight of the analogs. *C*log*P*, the log*P* values of the analogs calculated by the CLOGP program (parameter of lipophilicity), and the values for MM-C1P analogs were calculated using associate-ion structure, not free anion. NBD-C6 indicates an *N*-acyl C6 chain having an NBD group, and C1P-NBD indicates a C14-alkyl chain having an NBD group. The indicated cells were cultured with the medium with 5% serum in the presence of the indicated C1P analogs at 10 μM for 48 h. [Strong], cells appeared round with small vesicular-like specks within 6–12 h after treatment, and marked morphological changes were observed 24 h after stimulation in almost all cells. [Moderate], morphological changes were observed in 50% of cells examined at 24 h after the treatment. [Weak], slight and/or partial changes were observed 24 h after the treatment. [No], no morphological changes and no detachment within 48 h. [N.D.], not determined. Data are from two independent experiments. In the absence of serum, similar results were obtained 48 h after treatment with the C1P analogs.

24-well plates in the medium with serum. When the cells achieved 70–80% confluence (sub-confluent stage) at 24–48 h after seeding, they were used to examine the release of AA. The cells were labeled overnight with 0.05 μCi/mL of [^{3}H]AA in serum-free medium containing 0.1% fatty acid-free albumin (Sigma, A-7511) and 10 mM HEPES (pH 7.4). Washed cells were stimulated with various reagents such as PAF and PMA for the indicated periods at 37 °C. In some cases, the cells were pretreated with newly synthesized methyl-ester analogs of C1P for 30 min before the stimulation. The ^{3}H content of the supernatant was estimated, and data were calculated as percentages of all the radioactivities incorporated (20,000–30,000 dpm per well). The release of AA for 30 min without stimuli was 2–4% of the total incorporation in cells, and dependent on each experiment. In some cases, thus, the fold-increase was normalized as a percentage of the respective value without stimuli for quantitative analyses of the data.

2.4. Measurement of cPLA$_2$α activity

Human embryonic kidney cells (HEK293T cells) were transfected with an expression vector for human cPLA$_2$α [21] by LipofectAMINE PLUS (Invitrogen, Carlsbad, CA), according to the manufacturer's directions. The cells were homogenized with a Potter homogenizer in lysis buffer (0.34 M sucrose, 100 μM dithiothreitol, 0.2% CHAPS, and 10 mM HEPES, pH 7.4). cPLA$_2$α activity *in vitro* was measured using mixed micelles each containing 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine ([^{14}C]-PAPC, American Radiolabeled Chemicals, St. Louis, MO), Triton X-100 (Wako Pure Chemicals, Osaka, Japan), and the indicated compounds. The mixed lipids in the solvent (chloroform:methanol = 1: 1) were dried under nitrogen. A solution of 0.00125% Triton X-100 was added, and the lipid was vortexed vigorously for 2 min then sonicated for 5 min in a water bath. In some experiments, two vesicles containing [^{14}C]-PAPC and the C1P analogs were separately prepared. The assay buffer contained 100 mM HEPES (pH 7.4), 1 mg/mL of bovine serum albumin, 4 mM $CaCl_2$, and 10 mM dithiothreitol. The reaction (50 μL of the lipid vesicles solution and 175 μL of the assay buffer) was started by adding the enzyme sources (25 μL), and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated with Dole's reagent, and free fatty acid was recovered as described previously [7,22].

2.5. Uptake of fluorescent C6-ceramide and methyl-ester analogs of C1P in cells

The intracellular distribution of NBD-C6-ceramide (N-22651), BODIPY TR C5-ceramide (B-34400, Molecular Probes, Eugene, OR), and the newly synthesized analogs of DM-C1P having an NBD group were examined when cells achieved 40–50% confluence. Like NBD-C6-ceramide, NBD-C6-DM-C1P (Compound No **14**) has an NBD-labeled C6-*N*-acyl chain and a C18-alkyl chain. By contrast, C2-DM-C1P-NBD (**15**), C6-DM-C1P-NBD (**16**), and C16-DM-C1P-NBD (**17**) have an *N*-acyl chain of the indicated length and an NBD-labeled C14-alkyl chain. The intensity of the fluorescence of the NBD-labeled compounds was almost the same as that of NBD-C6-ceramide. CHO-W11A cells were incubated for the indicated periods with the indicated analogs. The distribution of fluorescence in cells was determined using a laser-scanning confocal microscope (Olympus, Tokyo), with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. For the analysis of lipids in cells, cells in a sub-confluent stage (12-well plates, 8×10^4 cells/well) were incubated with NBD-labeled compounds under the indicated conditions. Lipid extraction was performed using a chloroform/methanol solution followed by vigorous vortex mixing, and the lipids in the organic phase were analyzed on a TLC silica gel 60 plate (#105724, Merck, German) using 1-butanol:acetic acid:-water (3:1:1) as the mobile phase, as described [15,20]. Chemicals and organic solvents such as chloroform and methanol were purchased from Wako Pure Chemicals (Okasa, Japan). The fluorescence was detected with LAS1000-Plus (Fuji-Film, Tokyo; 470 nm excitation and 515 nm emission). The respective bands including the DM-C1P analogs having an NBD group, NBD-glucosylceramide, and NBD-sphingomyelin were identified based on reported Rf values [15,23,24]. In the lipid analysis, similar results were obtained in both conditions, at 30–40% confluence and in the sub-confluent stage.

2.6. Analyses of cell morphology and lactate dehydrogenase (LDH) leakage

The cells at 30–40% confluence and in the sub-confluent stage were used for analyses of morphological changes by phase contrast microscopy and LDH leakage, respectively. The quantification of cell viability was based on the LDH leakage method [25], and the cells were treated with the C1P analogs at 10 μM for 24 h. None of the analogs tested at 10 and 30 μM caused LDH leakage from L929 cells within 2 h after the stimulation.

2.7. Data presentation

Values for the release of AA from cells and the enzymatic activity of cPLA$_2$α are the mean ± S.E.M. for three or more

independent experiments performed in duplicate or triplicate. In some cases, data are shown as the mean ± S.D. of two or three determinations in a representative experiment. The data on uptake and metabolism of NBD-labeled reagents are the mean ± S.D. for the indicated number of independent experiments. In the case of multiple comparisons, the significance of differences was determined using a one-way analysis of variance with Dunnett's or Tukey's test. For pairwise comparisons, Student's two-tailed *t*-test was used. *P*-Values < 0.05 were considered to be significant. Morphological observations including the intracellular translocation of NBD-labeled reagents were representative results.

3. Results

3.1. Effects of mono- and di-methyl-ester analogs of C1P differing in N-acyl chain length on the release of AA mediated by cPLA$_2$α

C1P has been established to act as a stimulator of cPLA$_2$α both in assay using intact cells and in assay enzymatic activity [1,7,8]. First, we investigated the effects of methyl-ester analogs of C1P, newly synthesized in our laboratories (Fig. 1 and Table 1), on the release of AA from intact CHO-W11A cells. Neither MM-C1P analogs nor DM-C1P analogs at 10 μM released AA in 3 h (Fig. 2A) and 30 min (data not shown). Stimulation with 100 nM PAF (an agonist of PAF receptors) and with 1 μM A23187 plus 100 nM PMA (a Ca^{2+} ionophore and an activator of protein kinase C, respectively) has been established to release AA via cPLA$_2$α's activation [21,26]. The PAF-induced release of AA was significantly inhibited in the cells treated for 30 min with DM-C1P analogs having a short-*N*-acyl chain such as C2-DM-C1P (Compound No **1**), C5-DM-C1P (**2**), and C6-DM-C1P (**3**) (Fig. 2B). The inhibitory effect of these three analogs was concentration-dependent in the tested concentrations, although we could not examine the effects induced by the analogs at greater concentrations than 10 μM because of insolubility of the reagents to medium. In a typical experiment, 25, 45 and 60% inhibition in the 1, 3, and 10 μM C2-DM-C1P (**1**)-treated cells, and approximately 10, 40, and 45% inhibition in the 1, 3, and 10 μM C5-DM-C1P (**2**)- and C6-DM-C1P (**3**)-treated cells, respectively. Treatment with the DM-C1P analogs having a long-*N*-acyl chain and the MM-C1P analogs including C2-MM-C1P (**8**) and C6-MM-C1P (**10**) had no effect. The response to A23187/PMA was significantly inhibited by treatment with C2- (**1**), C5- (**2**), C6- (**3**), and C8-DM-C1P (**4**), but not with other DM-C1P analogs and MM-C1P analogs including C6-MM-C1P (**10**) (Fig. 2C). The release of AA induced by added secretory PLA$_2$ was not modified by DM-C1P analogs, and treatment with several MM-C1P analogs such as C2- (**8**) and C5-MM-C1P (**9**) enhanced the release (Fig. 2D). In subsequent experiments, we examined the inhibitory effects of DM-C1P analogs on the release of AA by cPLA$_2$α. The mechanism of the stimulatory effects of several MM-C1P analogs including C5-MM-C1P (**9**) on the secretory PLA$_2$-induced release of AA is currently being studied in our laboratory.

To examine whether DM-C1P analogs directly reduce the enzymatic activity of cPLA$_2$α, liposomes containing [^{14}C]-PAPC and the analogs were prepared and tested for cPLA$_2$α activity *in vitro*. The human cPLA$_2$α activity in the presence of PAPC liposomes alone was about 2,000 dpm/tube. The cPLA$_2$α activity in the liposomes, which combined PAPC with C5-DM-C1P (**2**) or C6-DM-C1P (**3**) at a molar ratio of 1:2.5, was significantly attenuated (Fig. 3). The existence of C16-DM-C1P (**6**) did not result in an inhibitory effect under these conditions. Previously, we reported that the cPLA$_2$α activity in liposomes which combined PAPC with sphingomyelin was markedly attenuated, but sphingomyelin vesicles generated separately from PAPC vesicles did not reduce the activity [22]. At a similar molar ratio, 1:2.5 (PAPC:DM-C1P analogs), the DM-C1P liposomes generated separately from the PAPC liposomes did not reduce cPLA$_2$α activity; the values were

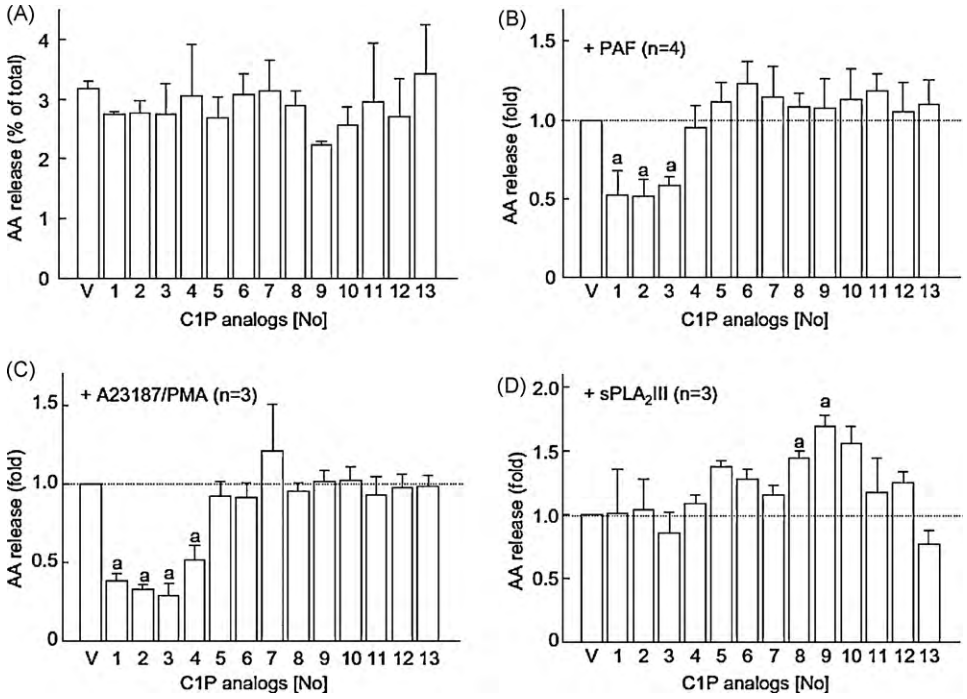

Fig. 2. Effects of methyl-ester analogs of C1P on the release of AA in CHO-W11A cells. In Panel A, [^{3}H]AA-labeled cells were stimulated with the indicated C1P analogs (shown by numbers, No) at 10 μM for 3 h. V, vehicle. Data are the mean ± S.D. of three determinations from a representative experiment repeated two times with similar results. The amount of AA released was expressed as a percentage of the total amount of AA incorporated. In Panels B–D, the cells were treated with the C1P analogs at 10 μM for 30 min, and then stimulated for 30 min with 100 nM PAF (B), 1 μM A23187 plus 100 nM PMA (C), or 1 μg/mL of type III secretory PLA$_2$ (D). Data are normalized as fold-increases of the values with respective stimuli, and are the mean ± S.E.M. for 3–4 independent experiments. The absolute values of the releases with vehicle were dependent on experiments (1.5–2.5% of total), and were 8.5 ± 1.7% ($n = 4$) with PAF, 20.3 ± 1.9% ($n = 3$) with A23187/PMA, and 4.1 ± 0.1% ($n = 3$) with secretory PLA$_2$. $^aP < 0.05$, significantly different from the control without the C1P analogs.

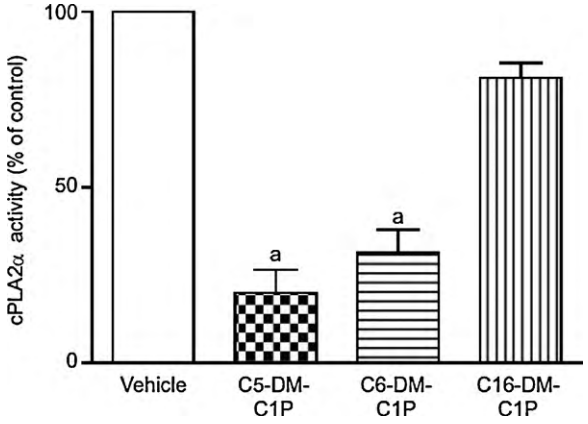

Fig. 3. Inhibition of cPLA$_2\alpha$ activity *in vitro* by DM-C5-C1P and DM-C6-C1P. The PLA$_2$ activity in the cytosolic fraction from HEK293T cells expressing human cPLA$_2\alpha$ was measured as described in Experimental Procedures. Data are normalized as a percentage of control, and are the mean ± S.E.M. for 3–4 independent experiments. [a] $P < 0.05$, significantly different from the control without the C1P analogs.

97.7 (% of control) with C5-DM-C1P (**2**), 95.2% with C6-DM-C1P (**3**), and 108.9% with C16-DM-C1P (**6**). These results showed that DM-C1P analogs having a short-*N*-acyl chain such as C6-DM-C1P (**3**) under the conditions tested reduced cPLA$_2\alpha$ activity probably by disturbing binding of the enzyme to glycerophospholipids such as PAPC, not by the trapping of cPLA$_2\alpha$ and/or direct binding of the enzyme.

3.2. Uptake, intracellular translocation, and metabolism of DM-C1P analogs having an NBD group

NBD-C6-ceramide, which has an NBD-labeled C6-*N*-acyl chain, has been established to be transported into cells, and a marker of the Golgi complex for visualization by fluorescence microscopy [27,28]. Under our conditions, NBD-C6-ceramide was accumulated in the Golgi complex in CHO-W11A cells at 30 min after the treatment (Fig. 4A). A marked accumulation of the fluorescence was observed at 2 h after the treatment, and this was obvious in the Golgi complex in the washed cells treated with NBD-C6-ceramide for 30 min and then incubated for 90 min in the NBD-C6-ceramide-free medium. NBD-C6-DM-C1P (**14**), which has an NBD-labeled C6-*N*-acyl chain

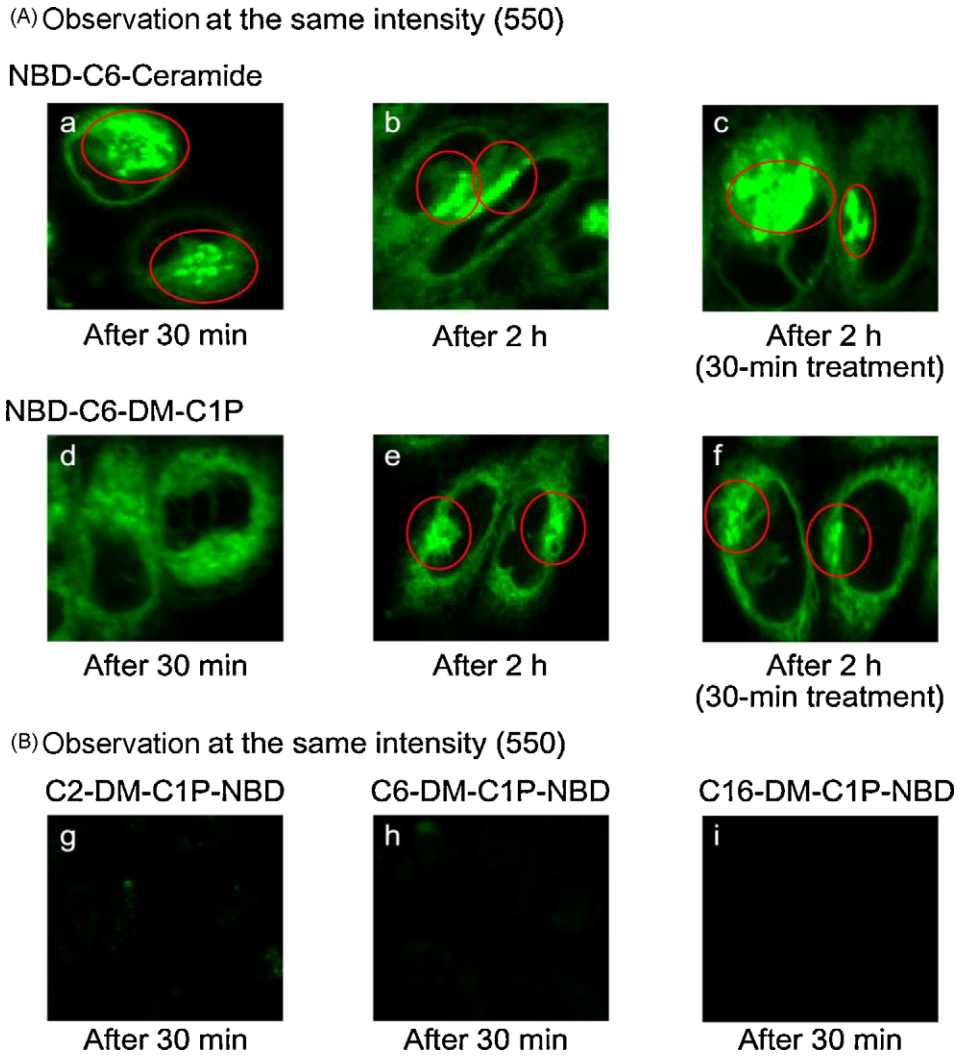

Fig. 4. The images of NBD-fluorescence in the cells treated with NBD-C6-ceramide and the DM-C1P analogs having an NBD group. In Panel A, CHO-W11A cells were incubated with NBD-C6-ceramide or NBD-C6-DM-C1P at 10 μM for 30 min (a and d) or for 2 h (b and e). In the right panels (c and f), the washed cells after the labeling with the indicated NBD compounds at 10 μM for 30 min were further cultured with the compound-free medium for an additional 90 min. Data are from a representative experiment repeated two or three times with similar results, and the images were obtained at the same intensity (PMT voltage, 550) in a fluorescence detector. In Panel B, cells were incubated with C2-DM-C1P-NBD (g), C6-DM-C1P-NBD (h), and C16-DM-C1P-NBD (i) at 10 μM for 30 min. The images in the cells treated with these analogs were marginally or not detected using the same intensity (PMT voltage, 550). Thus, the cellular distribution of the analogs was analyzed at an enhanced intensity (PMT voltage, 700–1000 shown in parentheses) in Panel C (j–r).

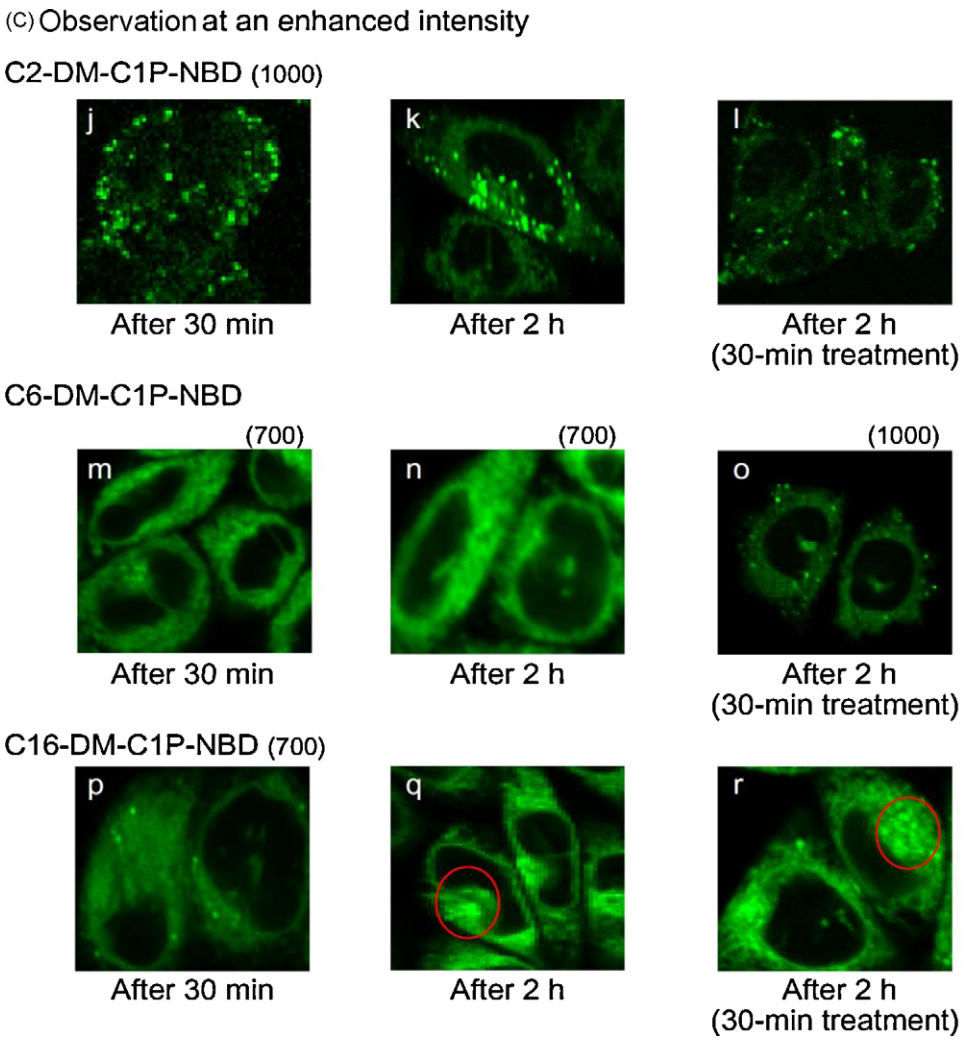

Fig. 4 (*Continued*).

and a DM-phosphate group, was taken up into cells to a degree and in a manner similar to NBD-C6-ceramide. The fluorescence derived from NBD-C6-DM-C1P (**14**) appeared to be obvious in the Golgi complex at 2 h even in the labeled and washed cells, and preferential localization of the compound in the Golgi complex was confirmed by fluorescent co-staining with BODIPY TR C5-ceramide, a marker of the Golgi complex (Supplementary Fig. 2). Next, we synthesized three C1P analogs that have an NBD-labeled C14-alkyl chain, an *N*-acyl chain of different lengths, and a DM-phosphate group; C2-DM-C1P-NBD (**15**), C6-DM-C1P-NBD (**16**), and C16-DM-C1P-NBD (**17**). Native cellular C1P derived from palmitoyl CoA and L-serine has a C18-alkyl chain, thus we synthesized C1P analogs having an NBD-labeled C14-alkyl chain taking the chemical length of an NBD group into consideration. These three analogs were taken up to a much lesser extent than NBD-C6-ceramide within 30 min (Fig. 4B) and 120 min (data not shown), and their characteristics are summarized in Table 2. In the enhanced detection system, the uptake of C2-DM-C1P-NBD (**15**) was not homogenous and the analog accumulated in vesicular-like specks (Fig. 4C). The uptake of C6- (**16**) and C16-DM-C1P-NBD (**17**) for 30 min was homogenous, and some of the compounds were accumulated in Golgi complex at 2 h after labeling (Fig. 4C and Supplementary Fig. 2). In the cells labeled with C6-DM-C1P-NBD (**16**) for 30 min and incubated for 90 min in the compound-free medium, the intensity of NBD fluorescence was decreased. By contrast, the intensity was retained in Golgi complex in the C16-DM-C1P-NBD (**17**)-labeled and washed cells at 2 h after the labeling.

As in the photo-image analysis (Fig. 4), the order for amounts of NBD-labeled compounds taken up for 30 min was; NBD-C6-ceramide > NBD-C6-DM-C1P (**14**) ≫ C6-DM-C1P-NBD (**16**) = C16-DM-C1P-NBD (**17**) > C2-DM-C1P-NBD (**15**), in the quantitative analysis (Fig. 5A). Next, we compared the cellular metabolism of NBD-C6-DM-C1P (**14**) and C6-DM-C1P-NBD (**16**) with that of NBD-C6-ceramide by using TLC. In CHO-W11A cells labeled with 10 μM NBD-C6-DM-C1P (**14**) for 30 min, there was a major band and a minor (under 5%) band (Fig. 5B). The Rf values of the major and minor bands were consistent with those of standard NBD-C6-DM-C1P (**14**) and NBD-C6-ceramide, respectively. The intensity of the two bands in cells incubated an additional 30 min (and 90 min, data not shown) decreased slightly (20%). In cells labeled with C6-DM-C1P-NBD (**16**), there were also two bands; a major C6-DM-C1P-NBD (**16**) band and a minor band (under 2%). The minor band appeared to be a C6-ceramide having an C14-alkyl chain with an NBD group, since it had a similar Rf value to that of NBD-C6-ceramide. Consistent with the results of the photo-image analysis (Fig. 4 and Table 2), the fluorescence of these bands was markedly decreased (under 10%) by additional incubation in the analog-free medium. There was no other detectable band in the cells labeled with NBD-C6-DM-C1P (**14**) or C6-DM-C1P-NBD (**16**). By contrast, NBD-C6-ceramide was metabolized to various molecules such as

Table 2
Uptake and localization of NBD-DM-C1P analogs in CHO-W11A cells.

NBD-C6-ceramide
30 min: 156 ± 5 pmol/well, marked uptake, specifically in Golgi complex.
2 h: Marked uptake, specifically in Golgi complex.
2 h (30-min labeling): Retained fluorescence, specifically in Golgi complex.
NBD-C6-DM-C1P (No **14**)
30 min: 103 ± 3 pmol/well, marked and homogenous uptake.
2 h: Marked uptake, specifically in Golgi complex.
2 h (30-min labeling): Retained fluorescence, specifically in Golgi complex.
C2-DM-C1P-NBD (No **15**)
30 min: 12.1 ± 0.4 pmol/well, marginal uptake in small vesicular specks.
2 h: Marginal uptake, specifically in small vesicular specks in specified areas.
2 h (30-min labeling): Lost fluorescence, some specks.
C6-DM-C1P-NBD (No **16**)
30 min: 40.4 ± 1.4 pmol/well, moderate and homogenous uptake.
2 h: Moderate and homogenous uptake, a slight condensation in Golgi complex.
2 h (30-min labeling): Lost fluorescence, some specks, not in Golgi complex.
C16-DM-C1P-NBD (No **17**)
30 min: 30.1 ± 1.1 pmol/well, moderate and homogenous uptake.
2 h: Moderate uptake, specifically in Golgi complex.
2 h (30-min labeling): Retained fluorescence, specifically in Golgi complex.

CHO-W11A cells were incubated with NBD-C6-ceramide or the indicated DM-C1P analogs with NBD at 10 μM for 30 min or 2 h. Absolute values of the indicated NBD-compounds taken up for 30 min per well (8×10^4 cells/well) are means ± S.D. for three independent experiments. In some cases (2 h, 30-min labeling), the cells were incubated with the indicated NBD compounds at 10 μM for 30 min, and then washed and further cultured with the compound-free medium for an additional 1.5 h. Data are from three independent experiments with similar results, and 50–70% of cells labeled with the NBD compounds showed similar characters in morphology in an experiment. Cells in a sub-confluent stage were used to measure the uptake of NBD-labeled compounds.

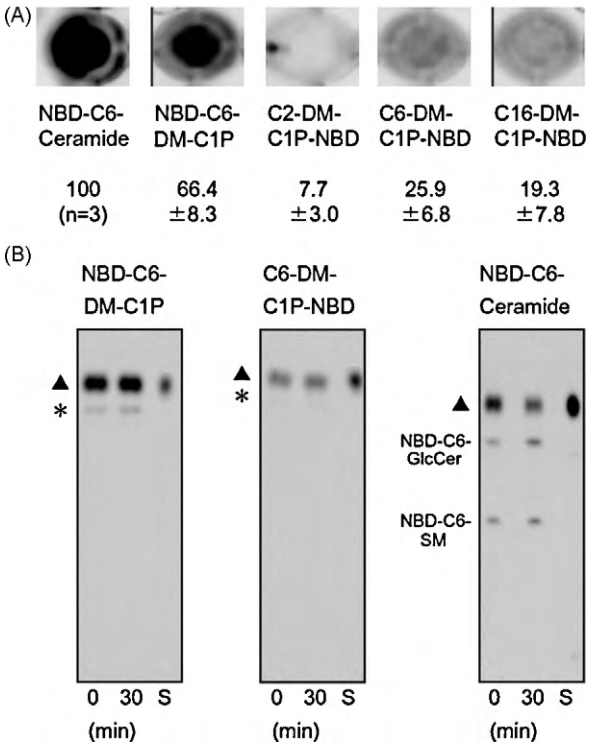

Fig. 5. Uptake and metabolism of DM-C1P analogs having an NBD group in cells. In Panels A and B, CHO-W11A cells were incubated with the indicated NBD-labeled compounds at 10 μM for 30 min, and the cellular lipids were extracted from the washed cells. The fluorescence derived from NBD-containing molecules was examined. Panel A shows typical images from an experiment. The quantitative data for the uptake for 30 min are shown as a percentage of the amount of NBD-C6-ceramide incorporated. The absolute values are shown in Table 2. In Panel B, cells were labeled with the indicated analogs at 10 μM for 30 min, and the washed cells (0 min) were further incubated for 30 min in analog-free buffer. The extracted lipids were separated by TLC: (▲) native reagent; (*) metabolized reagent; NBD-C6-GlcCer, NBD-C6-glucosylceramide; NBD-C6-SM, NBD-C6-sphymgomyelin; S, sample of a pure analog as a reference.

NBD-glucosylceramide and NBD-sphingomyelin in cells (Fig. 5B, Right Panel), as previously reported [15,20,23,24,28]. Like C2- (**1**) and C6-DM-C1P (**3**), both C2-DM-C1P-NBD (**15**) and C6-DM-C1P-NBD (**16**) inhibited the release of AA induced by PAF and by A23187/PMA, but not by secretory PLA_2, in CHO-W11A cells (Table 3). Treatment with C16-DM-C1P-NBD (**17**) had no effect on the release induced by the stimuli. These findings suggest that C6-DM-C1P (**3**) with and without an NBD group inhibited the release of AA via cPLA$_2\alpha$, and that the position of the NBD group on C6-DM-C1P (on the *N*-acyl chain (**14**) or alkyl chain (**16**)) did not affect C6-DM-C1P's actions on the release and cellular metabolism.

3.3. Morphological changes in cells by DM-C1P and MM-C1P analogs

L929 cells were treated with 10 μM of the analogs or vehicle containing 0.5% dimethyl sulfoxide (Fig. 6A). The cells treated with C5- (**2**) and C6-DM-C1P (**3**) appeared to be round at 24 h after the treatment. In some cases, the cell treated with these two analogs detached from dishes at 6 h after the treatment. Several small speck-like particles and/or vacuoles were observed in the C5- (**2**) and C6-DM-C1P (**3**)-treated cells by using differential interference contrast microscopy (data not shown), as observed in L929 cells exposed to oxidative stress [25]. Treatment with C2-DM-C1P (**1**) caused morphological changes in half of the cells and/or moderate morphological changes without detachment of cells at 24 h after the treatment (Table 1). Treatment with other C1P analogs including C16-DM-C1P (**6**), C5-MM-C1P (**9**), and C6-MM-C1P (**10**) had no effect on morphology. The responses in the absence of serum were almost the same as those shown in Table 1. Treatment with C2-C1P, C8-C1P, and C1P prepared from bovine brain did not cause marked morphological changes in L929 cells under the present conditions. Similar results were obtained in CHO-W11A cells treated with the C1P analogs (Panel B). In this cell line, the round cells were observed within 6–12 h after C5- (**2**) and C6-DM-C1P (**3**) treatment, and the morphology returned to normal at 48–72 h after treatment depending on the cells (data not shown). RLC-18 cells, a non-tumor cell line, are epithelial cells from rat liver [29]. Treatment with C5-DM-C1P (**2**) and C6-DM-C1P (**3**), but not C16-DM-C1P (**6**), caused morphological changes at 24 h after treatment (Panel C). The data concerning the C1P analogs tested in the present study are summarized in Table 1. A similar order of the analogs for cell toxicity was obtained in the LDH leakage assay, a quantitative marker of cell death, for 24 h after the treatment in L929 cells, although the responses were dependent on the experiment. In representative experiments ($n = 3$), values of LDH

Table 3
Effects of DM-C1P analogs having an NBD group on release of AA from cells.

	Release of AA (% of control)		
	PAF	A23187/PMA	Secretory PLA_2
Vehicle ($n = 5$)	100%	100%	100%
NBD-C6-DM-C1P ($n = 4$)	48.3 ± 2.2[a]	58.6 ± 2.8[a]	116 ± 10
C2-DM-C1P-NBD ($n = 3$)	62.2 ± 19.6	55.1 ± 11.9[a]	98.9 ± 13.9
C6-DM-C1P-NBD ($n = 3$)	58.9 ± 7.4[a]	66.9 ± 11.9[a]	142 ± 16
C16-DM-C1P-NBD ($n = 3$)	98.4 ± 22.6	68.9 ± 23.0	100 ± 13.9

[^{3}H]AA-labeled cells were stimulated with the indicated C1P analogs at 10 μM for 30 min, and then stimulated for 30 min with 100 nM PAF, 1 μM A23187 plus 100 nM PMA, or 1 μg/mL of type III secretory PLA_2. Data are normalized as fold-increases of the values with respective stimuli, and are the mean ± S.E.M. for the indicated number of independent experiments. Absolute values are described in Fig. 2.

[a] $P < 0.05$, significantly different from the control without the C1P analogs.

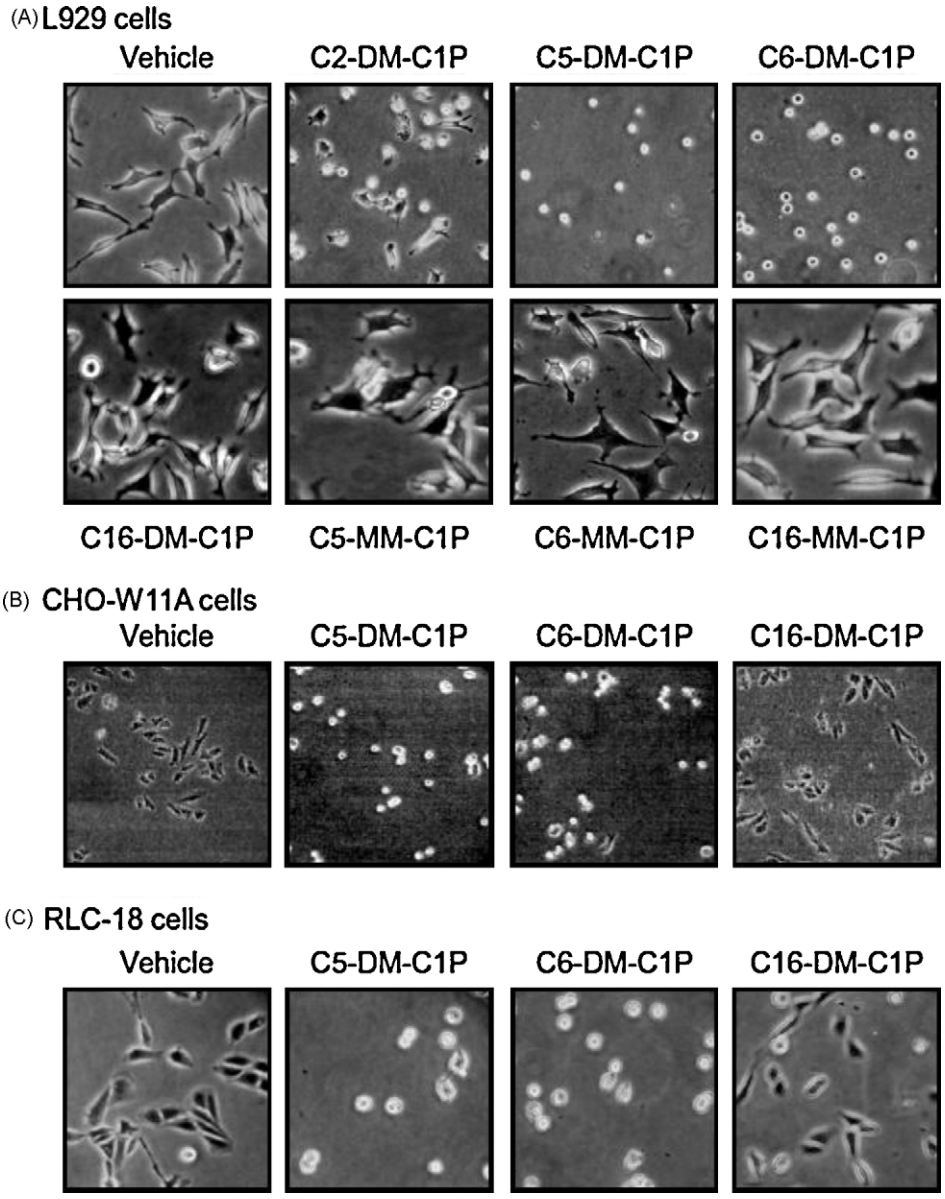

Fig. 6. Methyl-ester analogs of C1P induced morphological changes in various cell types. L929, CHO-W11A, and RLC-18 cells were cultured with vehicle or the indicated C1P analogs at 10 μM for 24 h, and morphological changes were observed by phase contrast microscopy. Data are from a representative experiment repeated three times with similar results. The data for the other C1P analogs tested are summarized in Table 1.

leakage were 5–8% in the vehicle-treated, 10–30% in the C2-DM-C1P (**1**)-treated, 30–60% in the C5-DM-C1P (**2**)-treated, 20–50% in the C6-DM-C1P (**3**)-treated, and 5–10% in the other C1P analog-treated cells, respectively. Like the analogs without an NBD group, C2-DM-C1P-NBD (**15**) and C6-DM-C1P-NBD (**16**) caused morphological changes including cell toxicity at 24 h after the treatment in the three cell types tested, and C16-DM-C1P-NBD (**17**) had no effect (Table 1).

4. Discussion

4.1. Effects of methyl-ester analogs of C1P on release of AA from cells and cPLA$_2$α activity

C1P has been shown to bind and activate cPLA$_2$α in many reports including ours [1,7,8]. Thus, the development of C1P analogs that can mimic and/or antagonize C1P's action may be useful for the control of release of AA and formation of eicosanoids. In the present study, we showed that DM-C1P analogs having a short-*N*-acyl chain, such as C2- (**1**), C5- (**2**), and C6-DM-C1P (**3**), but neither the DM-C1P analogs having a long-*N*-acyl chain nor MM-C1P analogs, inhibited the release of AA induced by PAF and A23187/PMA in CHO-W11A cells (Fig. 2), which was mediated by cPLA$_2$α's activation [21,26]. Consistent with the results obtained in intact cells, C5- (**2**) and C6-DM-C1P (**3**) inhibited human cPLA$_2$α activity *in vitro*, and C16-DM-C1P (**6**) had less of an effect on the activity (Fig. 3). The analogs including C6-DM-C1P (**3**) had no effect on the release of AA mediated by secretory PLA$_2$. Recently, we reported that sphingomyelin reduced cPLA$_2$α activity *in vitro* by disturbing the binding of the enzyme to substrate glycerophospholipids [22]. The data obtained using two preparations of liposomes suggest that the inhibition of cPLA$_2$α by C6-DM-C1P (**3**) is mediated by a similar mechanism. These results suggest that DM-C1P analogs having a short-*N*-acyl chain act as a selective inhibitor of cPLA$_2$α. The results were also supported by the following findings: (1) the existence of an NBD group on the C6-*N*-acyl chain, in addition to that on the alkyl chain, did not affect the action of C6-DM-C1P (**3**) as an inhibitor of cPLA$_2$α (Table 3) and (2)

C6-DM-C1P-NBD (**16**) and C16-DM-C1P-NBD (**17**) showed a similar uptake and intracellular distribution (Fig. 4C), but only C6-DM-C1P-NBD (**16**) inhibited the release of AA mediated by cPLA$_2\alpha$ (Table 3).

Wijesinghe et al. [10] reported that C1P-cPLA$_2\alpha$ interaction is structurally specific to the proper *N*-acyl chain length in C1P, and C2-C1P showed less of a stimulatory effect on the enzyme *in vitro*. In addition, a dimethyl-ester of C18:1-C1P did not stimulate cPLA$_2\alpha$ activity *in vitro* in their study. In the present study, similarly, DM-C1P analogs having a long-*N*-acyl chain including C16-DM-C1P (**6**) by themselves did not release AA in cells (Fig. 2) and C16-DM-C1P (**6**) did not activate cPLA$_2\alpha$ activity *in vitro* (Fig. 3). In contrast to C1P as an activator of cPLA$_2\alpha$, DM analogs of C1P having a short-*N*-acyl chain including C6-DM-C1P (**3**) showed greater effects on the enzyme. The obtained data did not show a linear dependency of *N*-acyl chain length on DM-C1P's actions (release of AA and cPLA$_2\alpha$ activity, Figs. 2 and 3), and NBD-C6-DM-C1P (**14**) and C6-DM-C1P-NBD (**16**) showed a similar potency although the exact length of *N*-acyl chain was different (Table 3). Our results, however, suggest that the length of the *N*-acyl chain of C1P analogs (C6- vs C16-*N*-acyl chains), in addition to the degree of methylation (DM- vs MM-esters) of a phosphate group, has a critical role in determining the potency of methyl-ester analogs of C1P as an inhibitor of cPLA$_2\alpha$. Further study concerning whether DM-C1P having a short-*N*-acyl chain inhibits cPLA$_2\alpha$ activity in the presence of C1P having a long-*N*-acyl chain including C16-C1P is needed.

4.2. Incorporation and metabolism of DM-C1P analogs

It is important to examine whether DM-C1P analogs are incorporated into intracellular spaces or not. As described in Section 1, the role of the uptake mechanism for C1P has been frequently discussed. Tauzin et al. [9] reported that the uptake of NBD-C6-C1P was less than 3% of that of NBD-C6-ceramide after 5 min, and only minute amounts of NBD-C6-C1P were found associated with the cells after 3 h. The migration of macrophages induced by C1P extracted from bovine brain appeared to be mediated by specific receptors that are coupled with pertussis toxin-sensitive G proteins and on plasma membranes [12]. By contrast, dispersed and/or sonicated preparations of C1P without organic solvents could induce various cellular responses [4,10,19,30]. Since the newly synthesized DM-C1P analogs are lipophilic compounds (log *P* values were greater than 5), they are likely to be incorporated into cells. In the present study, we synthesized several NBD-conjugated forms of DM-C1P and examined cellular uptake and translocation (Figs. 4 and 5). In sharp contrast to NBD-C6-C1P [9], NBD-C6-DM-C1P (**14**) which has an NBD group on the C6-*N*-acyl chain was effectively incorporated into cells, similar to NBD-C6-ceramide. By contrast, C6-DM-C1P-NBD (**16**) which has an NBD group on an alkyl chain, was taken up much less extensively than NBD-C6-DM-C1P (**14**) and NBD-C6-ceramide. Similarly, the uptake of both C2- (**15**) and C16-DM-C1P-NBD (**17**) was much lower than that of NBD-C6-DM-C1P (**14**). Our results showed that di-methylation of a phosphate group of C1P enhanced cell-permeability, and the position of the NBD group may determine the rate of uptake.

NBD-C6-ceramide was taken up into CHO-W11A cells and accumulated in the Golgi complex, where it was converted into various metabolites such as NBD-C6-sphingomyelin and NBD-C6-glucosylceramide (Fig. 5B), as reported [15,20]. The site for the synthesis of sphingomyelin and glucosylceramide is blocked by a DM-ester bond in DM-C1P analogs, thus the analogs are expected to be resistant to the production of these two metabolites. Consistent with this notion, C6-DM-C1P (**3**) analogs having an NBD group (both on the *N*-acyl chain and on the alkyl chain) were basically resistant to metabolism. Only a single type of metabolite was detected, and in small amounts (under 5%), for C6-DM-C1P analogs having an NBD group, and appeared to be ceramide forms of the analogs without a DM-phosphate group, as described in Section 3. Although NBD-caproic acid is formed via ceramidase activity in NBD-C6-ceramide-labeled cells [31], we did not detect NBD-caproic acid in the NBD-C6-DM-C1P (**14**)-treated cells (data not shown). Also, no metabolites corresponding to possible NBD-containing sphingosine-like molecules were detected in the lipids extracted from C6-DM-C1P-NBD (**16**)-treated cells (data not shown). Exogenous C18:1-C1P was slowly metabolized with only a small increase in C18:1-ceramide (less than 3% increase in total cellular ceramide content) for 2 h in A549 human lung adenocarcinoma cells [10]. Taking account of the limited uptake and stability of C6-DM-C1P-NBD (**16**) in cells, an intact C6-DM-C1P (**3**) (with or without an NBD group), not the metabolites including ceramide, appeared to inhibit release of AA via cPLA$_2\alpha$ at least within 2 h. Some DM-C1P analogs having an NBD group, like NBD-C6-ceramide, accumulated in Golgi complex, and the C6-DM-C1P-NBD (**16**) taken up appeared to be excluded to the medium (Fig. 4C). Some (including exclusion) system(s) in cells may function in the transport of DM-C1P analogs.

4.3. Cytotoxicity of DM-C1P analogs

Treatment with C5- (**2**) and C6-DM-C1P (**3**), but neither DM-C1P analogs having a long-*N*-acyl chain nor MM-C1P analogs, caused morphological changes and toxicity in the three types of cells tested. Treatment with C2-DM-C1P (**1**) showed moderate and/or weak cell toxicity, which may be explained by a limited incorporation and/or distinguished intracellular distribution of the reagent into cells (Fig. 4). These findings suggest that both the length of the *N*-acyl chain (C6- vs C16-*N*-acyl chains) and the number of methyl-esters (DM vs MM esters) in the C1P analogs are critical to the induction of cell toxicity, similar to the function as an inhibitor of cPLA$_2\alpha$. Previously, we reported that methyl-ester analogs of sphingosine-1-phosphate (S1P) caused cell toxicity dependent on their release of AA in L929 cells [11]. AA has been reported to show toxicity in various cell types including L929 cells [25,32]. In the present study, the DM-C1P analogs showing cell toxicity inhibited the release of AA, and did not stimulate the release, at least not over 3 h. Several clones of L929 cells lacking cPLA$_2\alpha$ due to RNA interference and a variant of L929 cells lacking cPLA$_2\alpha$ (C12 cells) showed normal cell growth [26]. Thus, the toxicity of C5- (**2**) and C6-DM-C1P (**3**) appeared to be independent on the inhibition of AA's release by these analogs at least in L929 cells.

No toxic effects of the C16-DM-C1P (**6**) analogs with and without NBD were observed in the three types of cells tested, although the cellular uptake and distribution of C6- (**16**) and C16-DM-C1P-NBD (**17**) were similar (Fig. 4). On morphological observation using the reagents at 10 μM, not only NBD-C6-ceramide and NBD-C6-DM-C1P (**14**) but also C6-DM-C1P-NBD (**16**) showed cell toxicity to the same degree with a similar time-dependency. Thus, the toxicity of DM-C1P analogs was dependent on the true length of the *N*-acyl chain, not on the uptake of C1P analogs or the presence and position of an NBD group. Ceramide has been well established to induce apoptosis and/or cell toxicity in various cell types [4,14,33]. However, amounts of the metabolites including C6-ceramide were quite limited in the CHO-W11A cells treated with 10 μM C6-DM-C1P (**3**) at least for 2 h as described in Section 3. Also, increasing the length of the *N*-acyl chain augmented the efficacy of ceramide as an inhibitor of the permeability transition pore and stimulator of apoptosis [34]. Thus, a possible role for ceramide in the cell toxicity induced by DM-C1P analogs may be excluded, although we could not exclude a role for additional metabolites in the cells treated for more than 24 h. Previously, we reported that dimethyl-ester analogs of S1P (D-*erythro*-*N*,*O*,*O*-trimethyl-S1P and L-*threo*-*O*,*O*-dimethyl-3*O*-benzyl-S1P) caused cell death in L929 cells in an

S1P receptor-independent manner [35]. The existence of a dimethyl-phosphate, not mono-methyl phosphate, group on C1P may be important for cell toxicity. Graf et al. [24] found that cells over-expressing ceramide kinase were sensitized to C2-ceramide-mediated apoptosis with a correlation with C2-C1P production. It was proposed that the accumulation of C1P having a short-*N*-acyl chain in cytoplasmic membrane leaflets leads to membrane fusion/budding resulting in changes in morphology and cell fate [24,36]. These findings may support our results showing the toxicity of DM-C1P analogs having a short-*N*-acyl chain. However, several investigators reported that C1P having a long-*N*-acyl chain showed toxicity in various cells [9,16,36,37]. Thus, the toxicity of the DM-C1P analogs having a short-*N*-acyl chain may be due to the intrinsic nature of the analogs. Precise mechanism of the DM-C1P analogs-induced cell toxicity including dependency of the length of *N*-acyl chain should be clarified.

4.4. Concluding remarks

In this study, we showed that newly synthesized dimethyl-ester analogs of C1P having a short-*N*-acyl chain including C6-DM-C1P (**3**) have two independent pharmacological effects; as an inhibitor of cPLA$_2\alpha$ and as an inducer of cell toxicity. The methyl-ester analogs of C1P are taken up and resistant to ceramide-related metabolism in cells. Also, our results suggest a potential role for ceramide/C1P analogs having a short-*N*-acyl chain as regulators of cellular responses. There are reports that short-*N*-acyl ceramides including C2-ceramide showed membrane-disrupting [38] and raft-destabilizing behavior [39,40]. Treatment with C2-ceramide inhibited the IgE/antigen-stimulated degranulation by decreasing the formation of long-*N*-acyl-C1P in mast cells [2]. As described in Section 1, C1Ps having a short-*N*-acyl chain can act as a mimetic of C1P or an exogenous agonist in some cases, although the analogs cannot always cause the same effects as C1P with a long-*N*-acyl chain [4,6,10,19,20,24]. These data including ours suggest that C1Ps having a short-*N*-acyl chain including C2-C1P are likely to modulate the responses induced by C1P having a long-*N*-acyl chain in cells. The newly synthesized lipophilic analogs of C1P having a short-*N*-acyl chain are likely to be useful for the regulation of ceramide- and C1P-related signaling and development of therapeutics for various AA metabolism-related diseases.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.07.028.

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