Cell-permeable ceramides preferentially inhibit coated vesicle formation and exocytosis in Chinese hamster ovary compared with Madin–Darby canine kidney cells by preventing the membrane association of ADP-ribosylation factor

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Differential effects of acetyl(C₂-) ceramide (*N*-acetylsphingosine) were studied on coated vesicle formation from Golgi-enriched membranes of Chinese hamster ovary (CHO) and Madin–Darby canine kidney (MDCK) cells. C₂-ceramide blocked the translocation of ADP-ribosylation factor-1 (ARF-1) and protein kinase C- α (PKC- α) to the membranes from CHO cells, but not those of MDCK cells. Consequently, C₂-ceramide blocked the stimulation of phospholipase D1 (PLD1) by the cytosol and guanosine 5'-[γ -thio]triphosphate (GTP[S]) in membranes from CHO cells. Basal specific activity of PLD1 and the concentration of ARF-1 were 3–4 times higher in Golgi-enriched membranes from MDCK cells compared with CHO cells. Moreover, PLD1 activity in MDCK cells was stimulated less by cytosol and GTP[S]. PLD2 was not detectable in the Golgi-enriched membranes. Incubation of intact CHO cells or their Golgi-enriched

membranes with C₂-ceramide also inhibited COP1 vesicle formation by membranes from CHO, but not MDCK, cells. Specificity was demonstrated, since dihydro-C₂-ceramide had no significant effect on ARF-1 translocation, PLD1 activation or vesicle formation in membranes from both cell types. C₂-ceramide also decreased the secretion of virus-like particles to a greater extent in CHO compared with MDCK cells, whereas dihydro-C₂-ceramide had no significant effect. The results demonstrate a biological effect of C₂-ceramide in CHO cells by decreasing ARF-1 and PKC- α binding to Golgi-enriched membranes, thereby preventing COP1 vesicle formation.

Key words: Golgi, phosphatidate, phospholipase D, protein kinase C, vesicle transport.

INTRODUCTION

In recent years there has been great interest in the roles of lipids, such as phosphoinositides and phosphatidic acid (PA), in regulating vesicle trafficking [1,2]. A major pathway for the formation of PA is via the action of phospholipase D (PLD; EC 3.1.4.4) on phosphatidylcholine (PC) [3]. The present work focuses on the role of another lipid mediator, ceramide, in membrane trafficking. Ceramides inhibit vesicle transport [4,5], but little is known about the mechanism for this effect. Our previous work with HL60 cells showed that ceramides can block the association of ADP-ribosylation factor (ARF) with membranes, thereby inhibiting the activation of PLD [6]. In this paper, we studied whether ceramides could block the formation of COP1-containing vesicles by interfering with the actions of ARF.

Hammond and co-workers [7,8] identified a human PLD (hPLD1a) and a shorter splice variant, hPLD1b, which are members of a highly conserved gene family. PLD1 is activated by protein kinase C (PKC)- α and - β isoforms, guanosine 5'-[γ -thio]triphosphate (GTP[S]) and small G-proteins, including ARF, RhoA and CDC42 [9]. Stimulation of PLD1 by small GTP-binding proteins and PKC isoforms is dependent on phosphatidylinositol 4,5-bisphosphate (PIP₂) [9]. PLD1 has been

reported to be associated with the endoplasmic reticulum, the Golgi complex, endosomes, secretory granules and lysosomes [1,7,10–13]. This distribution can also change upon cell activation. For example, 'priming' of neutrophils is accompanied by the relocation of PLD1 from secretory vesicles and the Golgi to the plasma membrane [14]. Another PLD isoform, PLD2, which also requires PIP₂ for activation, is located mainly in the plasma membrane [11,15,16]. PLD2 is not thought to be activated by ARF, Rho, PKC- α and PKC- β , although a small activation by ARF has been demonstrated recently [16].

The role of ARF in vesicle trafficking has been linked to PLD1 activation (for a review, see [1]). It was proposed that PLD activity and PA formation are required for the budding of COP1-containing vesicles from Golgi-enriched membranes [17,18]. The involvement of PLD was also supported by the observation that ethanol blocked COP1 vesicle formation in these membranes [17]. In addition, the requirement of ARF in coated vesicle formation was not pronounced in Golgi-enriched membranes from brefeldin A-insensitive cell lines that express constitutively high PLD1 activity [17]. PLD1 activity also appears to be involved in vesicle transport from the endoplasmic reticulum to the Golgi [19] and the release of nascent secretory vesicles from the *trans*-Golgi network [20–22]. It has also been proposed

Abbreviations used: ARF, ADP-ribosylation factor; C₂-, acetyl; CHO, Chinese hamster ovary; DAG, diacylglycerol; dihydro-C₂-ceramide, *N*-acetyldihydrosphingosine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GTP[S], guanosine 5'-[γ -thio]triphosphate; ([³H-methyl]PC), *sn*-1,2-dipalmitoylglycerophosphoryl[*N*-methyl-³H]choline; MDCK, Madin–Darby canine kidney; MEM- α , minimum Eagle's medium without nucleosides; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLD, phospholipase D; TNF α , tumour necrosis factor- α .

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that ARF-GTP and COP1 make up the minimum 'machinery' necessary to form COP-1 coated vesicles. ARF-1 enhances the PA pool that facilitates coatamer binding and directs the polymerization of the coat into a budding structure [18]. Jones and co-workers [1] concluded that available evidence does not support an essential role for PLD1 in the membrane recruitment of adaptor complexes, but that ARF could integrate downstream events. However, these authors also state that they cannot exclude the involvement of ARF-mediated PLD in the recruitment of adaptor complexes.

The present work was designed to study the mechanisms by which ceramides inhibit intracellular vesicle transport [4,5]. Ceramides are generated by acid and neutral sphingomyelinases via the actions of agonists, including tumour necrosis factor- α (TNF α), interleukin-1 and γ -interferon [23,24]. Ceramides act as second messengers to stimulate protein kinases [24] and phosphoprotein phosphatases [23]. Their generation is a key pathway in the inhibition of cell division, induction of cell differentiation and progression to apoptosis in many cell types [23,24]. In fibroblasts, ceramides activate a signalling cascade that involves the stimulation of tyrosine kinases, formation of Ras-GTP and activation of phosphoinositide 3-kinase and mitogen-activated protein kinase [25]. These pathways are involved in the stimulation of cell division and wound repair that is initiated by TNF α .

Our group also showed that acetyl- (C_2 -) and C_6 -ceramides inhibited the stimulation of PLD activity by a variety of growth factors in Rat2 fibroblasts [26,27]. Furthermore, C_2 -ceramide and sphingoid bases partially inhibited diacylglycerol formation by PLD in neutrophils [28], and C_6 -ceramide inhibited serum-stimulated diacylglycerol (DAG) accumulation and PLD activation in senescent cells [29]. Pre-treatment of mouse epidermal or human skin fibroblasts with sphingomyelinase or C_2 -ceramide specifically blocked the translocation of PKC- α , but not that of PKC- ϵ , to particulate fractions [30]. An inhibitory effect of C_2 -ceramide on PLD activation was also observed in rat basophilic leukaemia ('RBL-2H3') cells [31], and this was associated with an inhibition of the translocation of PKC- α , - β 1, - β 2 and - ϵ isoenzymes to membrane fractions. We showed in intact HL60 cells that C_2 -ceramide prevented the *N*-formylmethionyl-leucyl-phenylalanine-induced stimulation of PLD and decreased the amount of membrane-associated ARF1, RhoA, CDC42 PKC- α and PKC- β 1 [6].

In the present study we have investigated whether ceramides modify vesicle transport via effects on ARF and PLD activity. To accomplish this, we compared the relative effects of C_2 -ceramide on vesicle budding in Madin-Darby canine kidney (MDCK) cells, which express high, constitutive PLD activity and binding of ARF to membranes, with Chinese hamster ovary (CHO) cells, which have comparatively low amounts of membrane-bound ARF and PLD activity. C_2 -ceramide inhibited PLD1 activity, COP1 vesicle formation and viral particle secretion in CHO cells. These effects were related to an inhibition of ARF translocation to membranes. On the other hand, ceramides had a much smaller effect on coated vesicle formation and the secretion of viral particles in MDCK cells, which have higher basal levels of membrane-bound ARF. The results therefore identify a consequence of the action of ceramides in modifying the membrane association and activity of ARF.

EXPERIMENTAL

Materials

F-12 nutrient mixture (Ham's), Dulbecco's modified Eagle's medium (DMEM), minimum Eagle's medium without nucleosides (MEM- α), Hepes buffer, penicillin, streptomycin, and fetal

bovine serum (FBS) were obtained from Gibco BRL (Life Technologies, Burlington, ON, Canada). GTP[S] and PIP₂ were purchased from Roche (Laval, PQ, Canada). BSA and dialysed FBS were from Sigma (St Louis, MO, U.S.A.). *D-erythro*- C_2 -ceramide and *D-erythro*-dihydro- C_2 -ceramide (*N*-acetyldihydro-sphingosine) were from Biomol (Plymouth Meeting, PA, U.S.A.). L- α -PC (from bovine brain) and L- α -phosphatidylethanolamine (L- α -PE) were from Avanti Polar Lipids (Birmingham, AL, U.S.A.). PVDF and nitrocellulose membranes (0.45 μ m pore size) were purchased from Millipore Corp. (Bedford, MA, U.S.A.). Rabbit polyclonal anti-(β -COP) and anti-(PKC- α) antibodies were from Affinity Bioreagents, Inc. (Golden, CO, U.S.A.) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.) respectively. *sn*-1,2-Dipalmitoylglycerophosphoryl[*N*-methyl-³H]choline ([³H-methyl]PC) and enhanced chemiluminescence (ECL[®]) kits were from Amersham Pharmacia Biotech (Baie d'Urfé, PQ, Canada). DAG kinase was purchased from Calbiochem (San Diego, CA, U.S.A.). Anti-peptide human PLD1 [32] and anti-peptide human PLD2 antibodies [33], and rabbit antisera against ARF1 and ARF4 [34] were generously given by Dr S. Bourgoin (Université de Laval, PQ, Canada). C1 mouse anti-capsid antibody was provided by Dr Jerry Wolinski, (University of Texas, Houston, TX, U.S.A.). Goat anti-mouse horseradish-peroxidase-conjugated antibodies were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). Other reagents were of the highest quality available.

Cell culture

CHO and MDCK cells from the American Type Culture Collection (Manassas, VA, U.S.A.) were grown in either Ham's or DMEM medium, respectively, supplemented with 10% (v/v) heat-inactivated FBS, 100 units/ml penicillin and 100 μ g/ml streptomycin. Cell lines were maintained in a humidified atmosphere of 5% CO₂ at 37 °C, and they were passaged under subconfluent conditions. MDCK-24S cells were established [35] by stably transfecting MDCK cells with pRc/CMV-24S {a plasmid containing a G418 (or Geneticin) resistance gene and 24 S cDNA, which codes for the three rubella virus structural proteins, capsid and E2 and E1 glycoproteins [35]}. MDCK-24S cells were grown in DMEM containing 5% (v/v) FBS with antibiotics, 20 mM Hepes and 2 mM L-glutamine. CHO-24S cells were established as described previously [36], and were grown in media containing MEM- α , 10% (v/v) dialysed FBS, antibiotics, 20 mM Hepes and 2 mM L-glutamine. Both MDCK-24S and CHO-24S cells express rubella virus structural proteins, which form virus-like particles in the Golgi complex [36]. The virus particles are constitutively exocytosed and can therefore be used as a secretory marker.

Isolation of Golgi-enriched membranes and cytosolic fractions

The experiments were performed with Golgi-enriched membranes, which were isolated by the method of Balch and co-workers [37]. Cells from 100 mm diameter dishes were rinsed twice with ice-cold PBS, scraped from the dish and resuspended in 0.25 M sucrose containing 10 mM Tris/HCl, pH 7.4. Cell suspensions were then broken using a Dounce homogenizer, adjusted to 1.4 M sucrose, and overlaid with 1.2 and 0.8 M sucrose. The step gradients were centrifuged for 2 h at 28000 rev./min (141000 g_{max}) in an SW28 rotor (Beckman). Golgi-enriched membranes were collected from the 0.8 M/1.2 M sucrose interface by syringe puncture. Galactosyltransferase [38] and protein concentrations [6] were assayed as described previously. Where indicated, cells were pre-treated with 20 μ M

C₂-ceramide or dihydro-C₂-ceramide for 1 h, and the Golgi membranes were extracted as above. For the preparation of cytosol, cell homogenates in buffer A [90 mM KCl/50 mM Hepes (pH 7.2)] were centrifuged at 250 000 *g* for 60 min using a Beckman TL-100 ultracentrifuge. The supernatant was dialysed overnight at 4 °C with three changes of buffer A, adjusted to 4 mg/ml, frozen in liquid nitrogen and stored at -70 °C.

PLD assay

PLD activity was measured essentially as described by Aboualham and co-workers [6]. Golgi membranes (10 µg of protein) and cytosol (25 µg of protein) were pre-mixed at 4 °C in 70 µl, with 30 µM GTP[S] dissolved in 30 mM MgCl₂, 20 mM CaCl₂ and 400 mM NaCl, plus other additions, as specified. The substrate, which was composed of PE, PIP₂, PC (in molar proportions of 80:7:5 respectively) and [³H-methyl]PC (60 000 d.p.m. per assay), was freshly prepared in the form of phospholipid vesicles [39] and added in 30 µl to the assay mixture. The final concentrations of PIP₂ and PC in the assays were 12 and 8.6 µM respectively. Reactions were performed at 37 °C for 60 min stopped with methanol/chloroform (2:1, v/v), and lipid-extracted. [³H]Choline was separated from phosphoryl[³H]choline by using Dowex cation exchange resin, and was quantified by liquid-scintillation counting [6].

Formation of coated vesicles

Vesicle formation was measured essentially as described previously [17]. Golgi-enriched membranes (0.2 mg of protein/ml) were diluted with 5 vol. of buffer B [0.2 M sucrose/25 mM KCl/25 mM Hepes/2.5 mM MgSO₄/1 mM dithiothreitol (pH 7.2)], and then incubated in a total of 200 µl on ice. Cytosol (0.2 mg of protein) containing 30 µM GTP[S] was added to the membranes, and the samples were incubated at 37 °C for 15 min, after which they were centrifuged for 10 min at 12 000 *g* at 4 °C. Pellets were re-suspended gently in 200 µl of buffer B containing 0.25 M KCl, incubated on ice for 10 min and centrifuged, as described above. The resulting supernatants, which contained COP1-coated vesicles, were centrifuged at 250 000 *g* for 60 min using a Beckman TL-100 ultracentrifuge to pellet the vesicles. The pellets were re-suspended in Laemmli sample buffer, and then subjected to SDS/PAGE and immunoblotting with anti-βCOP, essentially as described previously [6].

Secretion of rubella virus-like particles

CHO-24S and MDCK-24S cells (7 × 10⁵) were seeded into 35 mm diameter dishes and grown overnight. The following day, cells were washed twice with PBS and incubated for 1 h with media containing DMSO, 40 µM C₂-ceramide or 40 µM dihydro-C₂-ceramide. The media were changed and the incubation was continued for 2 h. The media was aspirated and replaced with the equivalent fresh media and the incubation continued for a further 4 h. The media were collected and cleared of cell debris by centrifugation at 10 000 *g* for 5 min, and then rubella-like particles were pelleted at 100 000 *g* for 1 h at 4 °C using a Beckman TLA 120.2 rotor. Pellet fractions were analysed for the presence of rubella capsid protein by immunoblotting [40].

Protein electrophoresis and immunoblotting

Coated vesicles and Golgi membranes treated with cytosol and GTP[S] were dissolved in SDS sample buffer for 5 min at 90 °C, and proteins were separated by electrophoresis on 12% poly-

acrylamide gels [6]. Proteins were blotted on to PVDF membranes (Millipore) at 150 mA for 1 h, or overnight in the case of the β-COP subunit. Non-specific protein-binding sites were blocked by incubation for 1 h with 1% (w/v) BSA and 1% (w/v) non-fat dried milk in Tris-buffered saline. Western blot analysis was then performed using ECL[®] solutions (Amersham) following the manufacturer's recommendations, and quantified by densitometry at different exposure times to ensure a proportional response [6].

Measurement of ceramide and DAG concentrations

Concentrations of DAG and long-chain- and C₂-ceramides were measured by conversion into PA and ceramide 1-phosphates, respectively, by incubation with [³²P]ATP and DAG kinase, except that the incubation time was increased to 30 min. Results are expressed relative to the phospholipid content of the cells [6].

RESULTS

Effect of C₂-ceramide on the distribution of ARF1 and PKC-α in Golgi-enriched membranes of CHO and MDCK cells

The present study was designed to investigate whether C₂-ceramide would inhibit the formation of COP1 vesicles. For these studies, we used membrane fractions from CHO and MDCK cells that were enriched in Golgi markers, a model system used previously to study COP1 vesicle formation [17]. We postulated that ceramides might decrease COP1 vesicle formation by decreasing the binding of ARF to membranes. Furthermore, we predicted that the effects of C₂-ceramide should be more pronounced in membranes from CHO cells, which are expected to contain less membrane-bound ARF [17]. Our analysis showed that the ARF-1 concentration in Golgi-enriched membranes from CHO cells was 4-fold less than that for MDCK membranes (Figure 1A).

We then determined the effects of ceramides on the association of ARF-1 with membranes from the two cell types. Golgi-enriched membranes were incubated with cytosol and GTP[S], and then re-isolated by centrifugation (Figure 1B). In the presence of cytosol, GTP[S] induced the association of ARF-1 with Golgi membranes from CHO cells. This effect was less marked with Golgi membranes from MDCK cells. C₂-ceramide (20 µM) decreased the association of ARF-1 with Golgi membranes from CHO cells by approx. 85 ± 12% in three independent experiments. In contrast, 20 µM dihydro-C₂-ceramide had no significant effect (93 ± 25% ARF-1 binding relative to control). There was also no significant change in the amounts of ARF-1 that were associated with Golgi-enriched membranes from MDCK cells in the presence of GTP[S] (Figure 1B). The effects of C₂-ceramide on the association of PKC-α with the same membranes were also measured. C₂-ceramide (20 µM) decreased the association of PKC-α with Golgi-enriched membranes from CHO cells by approx. 35% in three independent experiments (Figure 1C). In contrast, 20 µM dihydro-C₂-ceramide had no significant effect. There was no significant effect of C₂-ceramide on the amount of PKC-α associated with Golgi-enriched membranes from MDCK cells.

Effect of C₂-ceramide on PLD1 activity in Golgi-enriched membranes of CHO and MDCK cells

We showed previously that ceramides inhibit PLD1 activity by preventing the translocation of ARF-1 and PKC-α to the membrane fractions of HL-60 cells [6]. A similar effect was observed in Figure 1 in membranes from CHO, but not MDCK,

Figure 1 Effect of ceramides on the distribution of ARF-1 and PKC-α in Golgi-enriched membranes

(A) Golgi-enriched membranes from the indicated cell lines were isolated, normalized for total protein, washed with 250 mM KCl, resuspended in buffer B and analysed for ARF content by Western blot analysis. The blot is representative of three independent experiments. (B and C) Golgi-enriched membranes were treated as in the assay for the formation of coated vesicles by using cytosol and GTP[S] in the presence of 20 μ M C₂-ceramide or dihydro-C₂-ceramide. Golgi-enriched membrane fractions from the pellet of the second centrifugation were recovered and analysed for ARF1 and PKC-α distribution. The blots are representative of three independent experiments. GTPγS, GTP[S].

Figure 2 Immunoblots of PLD1 and PLD2 from CHO and MDCK cells

Golgi membrane proteins (100 μ g) and total membrane proteins (100 μ g) from CHO and MDCK cells were separated on SDS/PAGE (10% gels), transferred to nitrocellulose membranes, and probed with the anti-PLD1 and anti-PLD2 antibodies. The blot illustrated is representative of two similar experiments.

cells. We therefore predicted that C₂-ceramide would have a greater effect in inhibiting PLD1 activation in membranes from CHO cells.

Our first objective was to characterize the distribution of PLD1 and PLD2 in total membranes and Golgi-enriched mem-

Figure 3 Effects of ceramides on PLD activity in Golgi-enriched membranes from CHO and MDCK cells

Golgi-enriched membranes (10 μ g of protein) and cytosol (25 μ g of protein) were assayed for PLD activity using a mixed liposome of PIP₂, PE and [³H]PC in the presence or the absence of GTP[S] (GTPγS). C₂-ceramide or dihydro-C₂-ceramide (20 μ M) was added to the reaction mixture, where indicated. Results are shown as means $\pm$ range from two independent experiments.

branes from both cell types using antibodies specific for PLD1 and PLD2 [32,33]. PLD1 was detected in total membrane fractions, and approx. 50 and 70 % of the PLD1 protein was recovered in Golgi-enriched membranes of CHO and MDCK cells respectively (Figure 2). The membrane fractionation process therefore resulted in the concentration of PLD1 with the Golgi marker in the membranes used for the experiments. PLD2 was also present in total membrane fractions from both cell types, but it was not detected in Golgi-enriched membrane fractions. Our results are therefore consistent with previous studies in which PLD2 was reported to be present mainly in plasma membranes [11,15,16].

PLD1 activity in Golgi-enriched membranes from CHO cells was stimulated approx. 4-fold when incubated with cytosol and GTP[S] (Figure 3). Addition of 20 μ M C₂-ceramide in the presence of albumin completely blocked this stimulation. In MDCK cells, the specific activity of the basal PLD1 activity in Golgi-enriched membranes was 2.5-fold higher than for the CHO membranes. This activity was only increased by approx. 20 % when incubated with cytosol and GTP[S]. Although C₂-ceramide decreased basal PLD1 activity in MDCK membranes and completely blocked stimulation by cytosol and GTP[S], the specific activity of PLD1 in the presence of C₂-ceramide was still approximately double that found in the CHO cells. Dihydro-C₂-ceramide (20 μ M) had no significant effect in either cell type. The higher PLD1 activity in the isolated Golgi membranes from MDCK compared with CHO cells was expected, on the basis of previous results [17].

Effect of ceramides on the formation of COP1 vesicles from Golgi-enriched membranes of CHO and MDCK cells

In the light of the relative effects of C₂-ceramide on the distribution of ARF and PLD activities shown in Figures 1 and 3, we predicted that C₂-ceramide would preferentially inhibit the formation of COP1 coated vesicles from membranes of CHO cells. We therefore adopted two different experimental approaches to investigate this hypothesis. In one type of experiment,

Figure 4 Effect of ceramides on the formation of coated vesicles from Golgi-enriched membranes of CHO and MDCK cells

Golgi-enriched membranes from CHO (A) or MDCK (B) cells were used to produce coated vesicles in the presence of 20 μ M C₂-ceramide or dihydro-C₂-ceramide. Alternatively, intact cells were treated with 20 μ M C₂-ceramide or dihydro-C₂-ceramide where indicated, and then Golgi-enriched membranes were isolated and assayed for coated vesicle formation, which was then assessed by Western blotting using anti-(β -COP) antibodies. A densitometric evaluation of the distribution of β -COP is shown. The 'control' condition, which is expressed as 100%, contained GTP[S] (GTP- γ S) without any added ceramides. Results are as means $\pm$ S.D. from three independent experiments.

20 μ M C₂-ceramide was added to Golgi-enriched membranes in the presence of cytosol and GTP[S], and the formation of COP1 vesicles was examined. In other experiments, intact CHO or MDCK cells were pre-treated with 20 μ M C₂-ceramide for 1 h, and then Golgi-enriched membranes were isolated and tested for their ability to form COP1 vesicles. Treatment of the cells with ceramide did not alter the recovery of Golgi-enriched membranes, as determined by measurement of galactosyltransferase activity (results not shown). In both types of experiment, C₂-ceramide, but not dihydro-C₂-ceramide, inhibited COP1 vesicle formation from Golgi-enriched membranes from CHO cells by approx. 60% and 52% respectively (Figure 4A). In contrast, COP1-coated vesicle formation from Golgi-enriched membranes of MDCK cells was not affected significantly by C₂-ceramide (Figure 4B). There was no significant formation of COP1-containing vesicles from the membrane fraction of either cell type in the absence of cytosol and GTP[S] (results not shown).

Table 1 Concentrations of DAGs and ceramides in CHO and MDCK cells

The concentrations of DAG and long-chain ceramides are shown relative to the phospholipid content in the two cell types. Cells were treated in the presence or absence of 40 μ M C₂-ceramide for 30 min as indicated. Results are means $\pm$ S.D. for three independent experiments. The significance of the differences between CHO and MDCK cells was calculated by using an unpaired *t* test as indicated by the asterisks (*). Differences within a cell type for C₂-ceramide treatment were evaluated with a paired *t* test, also as indicated ($\dagger$).

Lipid	Treatment	Cells ...	pmol of lipid per nmol of phospholipid	
			CHO	MDCK
DAG	None		10.5 $\pm$ 1.3	18.5 $\pm$ 9.1
	+ C ₂ -ceramide		17.4 $\pm$ 3.0	27.4 $\pm$ 10.8 $\dagger$
Long-chain ceramides	None		6.3 $\pm$ 0.9*	12.3 $\pm$ 2.4*
	+ C ₂ -ceramide		8.1 $\pm$ 0.9 $\dagger$	11.2 $\pm$ 0.5*

Figure 5 Effects of C₂-ceramide and dihydro-C₂-ceramide on the secretion of capsid proteins from CHO and MDCK cells

CHO-24S and MDCK-24S cells were pre-incubated for 2 h with 40 μ M C₂-ceramide or dihydro-C₂-ceramide and after changing the medium they were incubated for a further 4 h in the same medium. Rubella-like particles were collected after the second incubation and the level of capsid protein secretion was determined by Western blotting using C1 mouse anti-capsid antibodies. Results are expressed as means $\pm$ S.D. for three independent experiments.

Concentrations of DAG and ceramides in CHO and MDCK cells

To evaluate the differential effects of C₂-ceramide on PLD activity and COP1 vesicle formation in CHO and MDCK cells, we measured the concentrations of DAG and long-chain ceramides in cells that had been incubated in the presence or absence of C₂-ceramide (Table 1). The basal concentrations of long-chain ceramides, but not DAG, were higher in MDCK compared with CHO cells. Incubation with C₂-ceramide increased DAG concentrations in MDCK cells ($P = 0.022$, from a Student's paired *t* test), but this effect did not quite reach the necessary level for statistical significance in CHO cells ($P = 0.055$) with the limited numbers of independent experiments. Long-chain ceramide concentrations were increased after incubation with C₂-ceramide in the CHO, but not in the MDCK, cells. The concentration of C₂-ceramide associated with the CHO and MDCK cells after

incubation was 48 ± 9 and 39 ± 2 pmol/nmol of phospholipid (means $\pm$ ranges of two independent experiments) respectively.

Effects of C_2 -ceramide and dihydro- C_2 -ceramide on the secretion of capsid proteins from CHO and MDCK cells

The results so far indicate that C_2 -ceramide differentially affects the process of COP1 recruitment and vesicle formation with Golgi-enriched membrane fractions from CHO and MDCK cells. Since these processes are linked with the transport of itinerant proteins through the Golgi, we tested the effects of C_2 -ceramide on protein secretion from CHO and MDCK cells. Stably transfected CHO and MDCK cells that secrete rubella virus-like particles were used for these experiments. Virus particle assembly results in the secretion of rubella virus structural proteins into the culture medium [36]. CHO-24S and MDCK-24S cells were pre-incubated for 2 h with $40 \mu\text{M}$ C_2 -ceramide or dihydro- C_2 -ceramide and, after changing the medium, they were incubated for a further 4 h in the same medium. Rubella-like particles were collected after the second incubation, and the level of capsid protein was used to quantify secretion. In the CHO-24S cells, C_2 -ceramide decreased the secretion of capsid protein by 81 %, whereas the inhibition was only 53 % for MDCK-24S cells (Figure 5). Dihydro- C_2 -ceramide had no significant effect on the secretion of capsid protein, since the levels were 115 ± 20 % and 81 ± 14 % compared with the control incubations for CHO-24S and MDCK-24S cells respectively.

DISCUSSION

The present study examined mechanisms whereby ceramides could modulate intracellular transport and the formation of coated vesicles. This work was performed on the basis of our previous studies that showed that ceramides decrease the association of ARF, Rho, PKC- α and PKC- β with HL60 membranes and, consequently, inhibit PLD1 activity [6,26]. In the present study, we chose to compare the effects of C_2 -ceramide with Golgi-enriched membrane fractions from CHO and MDCK cells. Cytosol was used in these experiments since it contains the factors needed for COP1 vesicle formation. Golgi-enriched membranes from CHO cells were known to contain relatively less ARF than the equivalent membranes from MDCK cells, and to have lower basal PLD activity [10]. Our results confirmed these observations, and also showed that Golgi-enriched membranes from both cell types contained PLD1, but not PLD2. Furthermore, C_2 -ceramide blocked the translocation of ARF-1 to the membranes obtained from CHO cells to a much greater extent than that found with membranes from MDCK cells.

The biological consequences of this effect of ceramide were evaluated by measuring two 'downstream' actions of ARF; namely, PLD1 activation and COP1 vesicle formation. The relative inhibition of PLD1 activity by C_2 -ceramide was much more pronounced in membranes from CHO cells, which had lower PLD activity compared with MDCK cells. These differences in PLD1 activity and the effects of C_2 -ceramide were paralleled by the relative effects of C_2 -ceramide in blocking the translocation of the membranes of two PLD1 activators, ARF-1 and PKC- α . These latter two activators of PLD1 were chosen because of their involvement in vesicle transport from the Golgi apparatus (see below).

We also examined the effects of C_2 -ceramide on the formation of COP1-containing vesicles using two different approaches. Golgi-enriched membranes from CHO or MDCK cells were treated directly with ceramides or, alternatively, the cells were treated with ceramides before Golgi fractions were

subsequently isolated. Direct treatment of Golgi-enriched membranes with C_2 -ceramide mimics a situation that occurs after ceramide treatment of whole cells. In both protocols, the formation of coated vesicles was inhibited in the case of CHO cells, but not for MDCK cells. These results indicate that the major ceramide effect is direct, and that it does necessitate changes in intracellular signal transduction. In this respect, ceramide did not significantly alter the concentrations of cAMP in either cell type (results not shown). Also, we did not detect any significant effect of H89 (an inhibitor of PKA) or bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis-(acetoxymethyl ester) ('BAPTA/AM'; a chelator of intracellular Ca^{2+}) on the marked inhibition of capsid protein secretion in CHO cells (results not shown). We established the specificity of the C_2 -ceramide effect by demonstrating the lack of inhibition of PLD activity and COP1 vesicle formation with dihydro- C_2 -ceramide in Golgi-enriched membranes of CHO cells. Dihydro-derivatives of ceramides are often used as a control for ceramide action, but it should be noted that the physico-chemical properties of these derivatives differ from those of ceramides [41]. The relatively large accumulation of C_2 -ceramide that was observed in treated cells might therefore produce changes in membrane properties and fluidity that cannot be mimicked by dihydro- C_2 -ceramide. In spite of this, there were clear differences between the effects of C_2 -ceramide in CHO compared with MDCK cells.

ARF is well known to be involved in vesicle formation. Therefore the effects of C_2 -ceramide on COP1 vesicle formation in CHO cells might be explained by the ceramide-induced decrease in ARF-1 associated with the membrane fraction. The lower concentration of ARF in membranes of CHO cells cannot be explained by their having a higher content of long-chain ceramides compared with MDCK cells, since the reverse was the case (Table 1). It may be significant that incubation with C_2 -ceramide also increased the amounts of long-chain ceramide in the CHO cells, but not in MDCK cells. However, the changes in long-chain ceramide concentrations were small in comparison with the association of C_2 -ceramides with both cell types. Increases in long-chain ceramide concentrations have also been reported after incubating A431 human epidermoid carcinoma cells [42] and Rat2 fibroblasts [27] with short-chain ceramides, but the mechanism for this effect is not clearly established.

C_2 -ceramide decreased the association of PKC- α with membranes from CHO, but not MDCK, cells (Figure 1C). Other work has implicated PKC as a mediator of intracellular transport, including exocytosis [43], as well as receptor traffic through the endosomal system [44]. PKC- α activity is essential for the formation of constitutive transport vesicles at the *trans*-Golgi network, and may be involved in vesicle formation [45]. PKC- α and - ζ are associated with Golgi membranes [45]. In addition, PKC modulators affect the assembly of COP1 coats on Golgi cisternae [43]. We do not know the exact mechanism for the effect of C_2 -ceramide on the membrane association of PKC- α [6,29,30]. However, the results are compatible with the observed decrease in PLD1 activity (Figure 3) and an inhibitory effect of C_2 -ceramide in the formation of an active PLD1 complex involving ARF and PKC- α . Such a mechanism could explain why the effect of ceramide in membranes from CHO cells is dependent on GTP[S]. Furthermore, a ceramide-induced decrease in PA formation via PLD1 and the relatively low content of PA in the membranes of CHO cells [17] could result in a decreased formation of DAG needed for PKC- α translocation. Recruitment of membrane-bound PKC- α subsequent to PLD1 activation could serve as a positive feed-forward signal for enhancing PLD1 activation by ARF. Translocation of PKC- α is less marked in Golgi-enriched membranes from MDCK cells that contain

relatively more ARF and PKC- α under basal conditions (Figure 1C). However, this possible feed-forward signal via PKC- α need not occur if translocation depends upon polyunsaturated DAGs that may not be produced through the PLD pathway [46]. Whatever the exact mechanism for the C₂-ceramide effect on the translocation of PKC- α is, the decreased abundance of PKC- α in Golgi-enriched membranes from CHO cells could be important in producing the observed decrease in COP1 vesicle formation.

Results in Table 1 indicate that basal DAG concentrations were not significantly different between MDCK and CHO cells. Incubation of cells with C₂-ceramide increased DAG concentrations in MDCK cells, and this could involve the stimulation of PC:ceramide phosphocholine transferase activity. In CHO cells, the apparent increase in DAG did not quite reach the level of statistical significance. There was a very large accumulation of C₂-ceramide in both cell types, which could have a profound effect on membrane properties, as discussed above. This could be an important contributor towards preventing PKC activation.

The mechanisms whereby ARF stimulates vesicle transport appear to be cell-type-specific, and are controversial and not completely understood [1]. It has been proposed that PLD is involved in the formation of COP1 vesicles from Golgi-enriched membrane fractions of CHO cells, and this is compatible with the inhibition of vesicle formation by primary alcohols [17,47]. PLD1 activation involves the cytosolic factors ARF and PKC- α [48]. The differential effects of C₂-ceramide on the membrane association of these activators could explain the inhibition of PLD1 and thus COP1 vesicle formation in CHO, but not in MDCK, cells. COP1 vesicle formation probably reflects transport between the Golgi and other membranes, such as the endoplasmic reticulum. Our studies demonstrate that PLD1 is concentrated in the Golgi-enriched membranes from CHO and MDCK cells, and that there was no detectable PLD2 protein. These results do not exclude the possibility that PLD1 could also be located in membranes derived from the endoplasmic reticulum, endosomes, lysosomes and secretory granules [1,7,10–13]. However, the inhibition of COP1 vesicle transport by primary alcohols in CHO cells [17] provides a strong argument that ARF-dependent PLD1 activity is involved in promoting the formation of these vesicles from Golgi membranes in this system.

An alternative explanation for the action of ARF is that it controls vesicle formation independently of PLD activation [1,49–52]. It is thought that PIP₂ may be a 'common denominator' molecule that is responsible for vesicle budding mediated by ARF and phosphatidylinositol transport protein [1]. PLD1 activity is normally low under basal conditions, and it may not be involved in constitutive vesicle trafficking. Nevertheless, PLD1 could gain in importance after receptor-mediated cell stimulation, resulting in increased vesicle movement. In this respect, the formation of PA could activate phosphatidylinositol 4-phosphate 5-kinase [53] and the formation of PIP₂. New synthesis of PIP₂ is unlikely to explain the present results, since no ATP was added to the membrane fractions during the incubations. We had shown previously that inhibition of PLD activity by ceramides is independent of ATP [6]. However, the ceramide inhibition was attenuated by adding PIP₂ to the incubations [6]. Our more recent work shows that ceramides interact reversibly with the catalytic cores of mammalian PLD1 and PLD2, causing their inhibition [54]. This effect is mediated directly, and does not require the intervention of signal-transduction pathways. The inhibition by ceramide of PLD1 and PLD2 is competitive with the concentration of PIP₂, which activates both isoforms. Therefore ceramide inhibition of PLD1 and PLD2 in different cells could also be modulated by differences in PIP₂ concentrations. Our present results demonstrate that C₂-

ceramide inhibits the interaction of ARF with membranes of CHO cells, and that this could therefore block the stimulatory effects of ARF on COP1 vesicle budding in intact cells by both PLD-dependent and -independent mechanisms. In contrast, membrane-bound ARF1 concentrations were higher in MDCK cells, and C₂-ceramide failed to either displace ARF from membranes or inhibit COP1 vesicle formation.

We also investigated the action of C₂-ceramide on the secretion of proteins through exocytosis. This was achieved by using both CHO-24S and MDCK-24S cells that constitutively secrete rubella virus structural proteins. The process of exocytosis is coupled with COP1 budding in the Golgi complex. Viral protein secretion was also more strongly inhibited by C₂-ceramide in CHO compared with MDCK cells, although there was still a significant level of inhibition in the MDCK cells. Dihydro-C₂-ceramide had no significant effect in either cell line, which demonstrates a specificity of the ceramide effect. Ceramide also inhibits the secretion of ³⁵S-labelled proteins from HL-60 cells [55] and slows anterograde traffic in BHK-21 cells [56]. Furthermore, Rosenwald and Pagano [4] showed that incubation of CHO cells that were infected with the vesicular stomatitis virus with C₆-ceramide decreased the rate of viral glycoprotein transport through the Golgi complex, and reduced the release of infectious virions. The mechanism by which ceramide exerts these effects was not clarified by Rosenwald and Pagano [4], and they did not compare the effect of ceramide action in CHO cells with MDCK cells. Our results indicate that the ceramide inhibition of protein secretion involves preferential inhibition of ARF association with membranes in CHO cells.

We also treated CHO and MDCK cells for 30 min with 10 ng/ml of TNF α to test whether this would alter ceramide concentrations. However, we failed to detect a significant increase in ceramide concentrations after this relatively short incubation, and there was no significant decrease in the secretion of capsid protein (results not shown). We therefore did not demonstrate under the present conditions that ceramide formation induced by a physiological agonist could produce the same effects as C₂-ceramide. Other work has previously documented relatively small increases in ceramide concentrations in cells after short-term treatment. The larger increases in ceramide concentrations, that tend to be associated with apoptosis, occur after approx. 10 h [23]. In contrast, we demonstrated in studies with Rat2 fibroblasts that TNF α treatment for 1 h showed similar effects to those resulting from the addition of sphingomyelinase or C₂-ceramide on the formation of actin stress fibres [57]. In these fibroblasts, all three agonists increased the membrane association and activation of the small G-proteins Cdc42, Rac and Rho. This depended on the prior activation of Ras, phosphoinositide 3-kinase and sphingosine kinase [25,57]. Such a signalling cascade is a reflection of the ability of TNF α to stimulate the division of fibroblasts, rather than to cause apoptosis, as it does in many cell types. The more direct effects of C₂-ceramide in inhibiting the membrane association of Cdc42, Rho and ARF1 that were observed by Abousalham and co-workers [6], and those for ARF in the present study, could result from ceramide-induced changes in membrane properties, and clearly differ from those in Rat2 fibroblasts. Although ceramides themselves may cause activation of various G-proteins in some cells, they also appear to decrease the effectiveness of other agonists in causing these activations [6,57].

In conclusion, our work provides novel evidence that ceramides, when added to intact CHO cells and their Golgi-enriched membranes, can decrease COP1 vesicle production by means of their ability to block the translocation of ARF and PKC- α to membranes. The present results therefore provide further mech-

anistic information concerning the actions of ceramides in inhibiting membrane transport, and demonstrate that these effects are cell-type-specific.

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