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Inhibition of arachidonic acid release and cytosolic phospholipase $A_2\alpha$ activity by D-*erythro*-sphingosine

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Abstract

Sphingolipid metabolites such as sphingosine 1-phosphate (S1P) and ceramide can mediate many cellular events including apoptosis, stress responses and growth arrest. Although ceramide stimulates arachidonic acid metabolism in several cells, the effects of sphingosine and its endogenous analogs have not been established. We investigated the effects of D-*erythro*-sphingosine and its metabolites on arachidonic acid release in the two cells and on the activity of cytosolic phospholipase $A_2\alpha$. C2-Ceramide (*N*-acetyl-D-*erythro*-sphingosine, 100 μM) alone stimulated [^{3}H]arachidonic acid release and enhanced the ionomycin-induced release from the prelabeled PC12 cells and L929 cells. In contrast, exogenous addition of D-*erythro*-sphingosine inhibited the responses in a concentration-dependent manner in the two cell lines. D-*erythro*-sphingosine, D-*erythro*-*N,N*-dimethylsphingosine (D-*erythro*-DMS) and D-*erythro*-dihydrosphingosine (D-*erythro*-DHS) significantly inhibited mastoparan-, but not Na_3VO_4-, stimulated arachidonic acid release in PC12 cells. D-*erythro*-S1P and DL-*threo*-DHS showed no effect on the responses. Production of prostaglandin $F_{2\alpha}$ was also enhanced by C2-ceramide (20 μM) and suppressed by D-*erythro*-sphingosine (10 μM) in PC12 cells. An in vitro study revealed that D-*erythro*-sphingosine, D-*erythro*-DMS and D-*erythro*-DHS directly inhibited cytosolic phospholipase $A_2\alpha$ activity. These findings suggest that ceramide and D-*erythro*-analogs of sphingosine have opposite effects on phospholipase A_2 activity and thus regulate arachidonic acid release from cells.

Keywords: D-*erythro*-sphingosine; D-*erythro*-dihydrosphingosine; D-*erythro*-*N,N*-dimethylsphingosine; Arachidonic acid; Phospholipase A_2; PC12 cell

1. Introduction

Ceramide and its intermediate breakdown products (sphingolipid metabolites) are involved in various cellular events such as apoptosis, stress responses, and growth arrest (Hannun, 1996; Levade and Jaffrézou, 1999; Pyne and Pyne, 2000). Ceramide is produced by de novo synthesis or in response to stress or agonists from sphingomyeline by the activation of sphingomyelinases, and is metabolized to sphingosine by ceramidases. Sphingosine is phosphorylated by sphingosine kinases to sphingosine 1-phosphate (S1P), and can also be *N*-methylated to D-*erythro*-*N,N*-dimethylsphingosine (D-*erythro*-DMS) in cells and tissues (Igarashi, 1997; Spiegel, 1999). D-*erythro*-Dihydrosphingosine (D-*erythro*-DHS) is a processor in de novo synthesis of ceramide and a substrate for sphingosine kinases. These sphingosine metabolites play important roles in the regulation of mitogenesis, differentiation, cell death and cycle arrest, so on (Hannun, 1996; Igarashi, 1997; Spiegel, 1999; Pyne and Pyne, 2000). Although D-*erythro*-S1P is known to be released to extracellular spaces and then act as endogenous agonist for the EDG (endothelial differentiation gene) family of G protein-coupled cell surface receptors in cells, the sphingosine metabolites including S1P can act as intracellular second messengers in various cell types (Hannun, 1996; Spiegel, 1999; Pyne and Pyne, 2000). In many cases, however, how sphingosine metabolites regulate activity of signaling molecules remains to be elucidated.

Stimulation of tumor necrosis factor receptor increased ceramide levels, cytosolic phospholipase A_2 ($cPLA_2$) activity and cell death in L929 cells (Hayakawa et al., 1993; Wiegmann et al., 1994). In addition, $cPLA_2$ is a necessary component in sphingolipid metabolism such as ceramide

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accumulation and cell death (Jayadev et al., 1997), and the ratio of arachidonic acid in phospholipids can represent an important signal leading to cell death (Surette et al., 1996). Cell permeable C6-ceramide (*N*-hexanoylsphingosine) accelerated Ca^{2+}-dependent translocation of $cPLA_2$ to the membrane fraction in platelets (Kitatani et al., 2000). C6-ceramide and sphingosine, which alone showed no effect, enhanced thromboxane A_2 analog-stimulated arachidonic acid release in rabbit platelets (Hashizume et al., 1997, 1999; Sato et al.,1999). In contrast, it was reported that C6-ceramide prevented $cPLA_2$ and production of prostaglandin D_2 in a mast cell line (Kitatani et al., 2001), and sphingosine inhibited PLA_2 activities in various tissues such as neutrophils in vitro (Franson et al., 1992). Thus, the effects of sphingolipid metabolites such as ceramide and sphingosine on arachidonic acid release and PLA_2 activity have not been well established.

In pheochromocytoma PC12 cells, sphingolipid metabolites showed various responses. Ceramide induced an increase in cytosolic and mitochondrial free Ca^{2+} concentrations and apoptosis (Hartfield et al., 1997; Yoshimura et al., 1998; Darios et al., 2003). Although exogenous addition of D-*erythro*-S1P caused cell rounding and neurite retraction via cell surface receptors, probably via EDG-5 (Sato et al., 1997; MacLennan et al., 1999; Van Brocklyn et al., 1999), S1P appeared to act intracellularly to protect against apoptosis in PC12 cells (Edsall et al., 1997, 2001; Van Brocklyn et al., 1998; Olivera et al., 1999). In the present study, we investigated the effects of sphingolipid metabolites on arachidonic acid release in L929 and PC12 cells, and on the activity of $cPLA_2\alpha$ in vitro. We propose that D-*erythro*-sphingosine and its endogenous analogs, but not D-*erythro*-S1P, act as inhibitors of arachidonic acid release and/or PLA_2 activity.

2. Experimental procedures

2.1. Materials

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (215 Ci/mmol, 7.96 TBq/mmol) and 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine were purchased from Amersham (Buckinghamshire, UK) and Perkin Elmer (Boston, MA, USA), respectively. C2-Ceramide (D-*erythro*-*N*-acetylsphingosine), D-*erythro*-sphingosine, D-*erythro*-DHS, D-*erythro*-DMS, DL-*threo*-dihydrosphingosine (DL-*threo*-DHS), ionomycin, mastoparan, phorbol myristate acetate and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) were obtained from Sigma (St. Louis, MO, USA). D-*erythro*-S1P and 1,6-bis(cyclohexyloximinocarbonyl-amino)hexane (RHC80275) were purchased from Biomol Res. Lab. (Plymouth Meeting, PA, USA). 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Gö6976) and 1.4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126) were from Calbiochem (La Jolla, CA, USA) and Promega (Woods Hallow, WI, USA), respectively. Bromoenol lactone was from Cayman (Ann Arbor, MI, USA).

2.2. Cell cultures and [^{3}H]arachidonic acid release from cells

PC12 cells (a rat pheochromocytoma cell line) were cultured on collagen-coated dishes in Dulbecco's modified Eagle's (DME) medium supplemented with 5% heat-inactivated fetal bovine serum and 5% horse serum, as described previously (Thang et al., 2000; Someya et al., 2002). L929 cells (a murine fibrosarcoma cell line) were cultured in the DME medium supplemented with 5% heat-inactivated fetal bovine serum (Hayakawa et al., 1993). [^{3}H]Arachidonic acid release from the prelabeled cells was determined as described previously (Mori et al., 2001; Someya et al., 2002). In brief, subconfluent PC12 cells or L929 cells on dishes were prelabed with 0.33 μCi (12.2 kBq/ml) of [^{3}H]arachidonic acid for 24 h in the DME medium containing 0.2% serum. The cells were washed and suspended in modified Tyrode HEPES buffer (137 mM NaCl, 5 mM KCl, 5 mM glucose, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES, pH 7.4). Cell suspensions (30–50 μg protein) were incubated with the indicated agents for 30 min at 37 °C in the presence of 0.1% fatty acid-free bovine serum albumin (Sigma A-7511). In some experiments, cell suspensions were prepared with the $CaCl_2$-free buffer, and [^{3}H]arachidonic acid release was measured in the $CaCl_2$-free buffer containing 0.2 mM EGTA. The total volume was 200 μl and the reaction was terminated by the addition of 500 μl of ice-cold, Ca^{2+}-free, Mg^{2+}-free Tyrode buffer containing 5 mM EDTA and EGTA followed by centrifugation (5000 × *g*, 30 s) at 4 °C. The amount of the ^{3}H radioactivity released into the supernatant was expressed as a percentage of the total incorporated radioactivity (15,000–20,000 dpm per tube).

2.3. Measurements of intracellular free Ca^{2+} concentrations and lactate dehydrogenase leakage in PC12 cells

Intracellular free Ca^{2+} concentrations in PC12 cells were determined as described previously (Murayama et al., 1995). Cell viability was estimated by the leakage of lactate dehydrogenase as described previously (Yasuda et al., 1999). The leakage (%) was defined as the ratio of lactate dehydrogenase activity in the culture medium and total activity [%=(extracellular activity)/(extracellular activity and remaining cellular activity) × 100] per well.

2.4. Expression of $cPLA_2\alpha$ in human embryonic kidney (HEK) 293T cells and PLA_2 activity in vitro

HEK293T cells were transfected with pcDNA4/HisMax A-human $cPLA_2\alpha$ by LipofectAMINE (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols.

The expression of cPLA$_2\alpha$ was confirmed by immunoblotting using anti-cPLA$_2\alpha$ antibody (Santa Cruz, N-216, Santa Cruz, CA, USA). PLA$_2$ activity was measured using 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine as the substrate as previously described (Muthalif et al., 1996). Briefly, 50 μl of 10 μM radiolabeled phospholipid (~ 6000 dpm per tube) sonicated in 0.1% Triton X-100 was added to 25 μl of the cell lysate fractions (12.5 μg protein) and 175 μl of reaction buffer (50 mM HEPES, 1 mg/ml of bovine serum albumin, 4 mM $CaCl_2$, 10 mM dithiothreitol, pH 7.4). The reaction mixture (total 250 μl) was incubated at 37 °C for 30 min. The reaction was stopped by adding 1.25 ml of Dole's reagent (1N H_2SO_4/*n*-heptane/isopropanol = 2/20/78 ratio). Then 0.75 ml of n-heptane and 0.5 ml of water were added and centrifugated (5000 × *g*, 5 min). The supernatant (0.75 ml) was collected and 0.75 ml of *n*-heptane and 100 mg of silica were added to the supernatant. After the centrifugation, the radioactivity in the *n*-heptane phase containing [^{14}C]arachidonic acid was quantified.

2.5. Prostaglandin $F_{2\alpha}$ formation in PC12 cells

PC12 cell suspensions were incubated with the DME medium containing 0.1% fatty acid-free albumin at 37 °C in the presence of the indicated agents. The contents of prostaglandin $F_{2\alpha}$ in the supernatant after centrifugation (1000 × *g*, 30 s, 4 °C) were determined using an enzyme immunoassay kit (Cayman). Although 100 μM (30 μg/ml) sphingosine solution without PC12 cells cross-reacted with the kit slightly, the 10 μM sphingosine did not show a significant cross-reactivity.

2.6. Statistics

Values are means ± S.E.M. for three to four independent experiments performed in triplicate assays. In the case of multiple comparisons, the significance of differences was determined using one-way analysis of variance by Dunnett's or Tukey's test. For pairwise comparisons, Student's two-tailed *t*-test was used. *P* values at <0.05 were considered to be significant.

3. Results

3.1. Effects of C2-ceramide and D-erythro-sphingosine analogs on [^{3}H]arachidonic acid release in PC12 cells and L929 cells

The addition of cell permeable C2-ceramide at 100 μM significantly stimulated [^{3}H]arachidonic acid release from the prelabeled PC12 cells in the presence of 2 mM $CaCl_2$ (Table 1). The effect of C2-ceramide was significant at 10 min, and reached a plateau at 20–30 min after addition. D-*erythro*-Sphingosine, D-*erythro*-DMS and D-*erythro*-DHS at 100 μM slightly inhibited [^{3}H]arachidonic acid release, and 5 μM ionomycin alone stimulated the release significantly. In the presence of 5 μM ionomycin, 100 μM C2-ceramide stimulated the release, but D-*erythro*-sphingosine, D-*erythro*-DMS and D-*erythro*-DHS (100 μM, respectively) inhibited the release. Addition of 10 μM (data not shown) and 100 μM D-*eryhtro*-S1P had no effect with and without 5 μM ionomycin. In addition, DL-*threo*-DHS showed marginal effects with and without ionomycin. Addition of D-*erythro*-sphingosine and D-*erythro*-DHS, but neither D-*erythro*-DMS, D-*eryhtro*-S1P or DL-*threo*-DHS, inhibited [^{3}H]arachidonic acid release induced by 100 μM C2-ceramide plus 5 μM ionomycin in a concentration-dependent manner (Fig. 1). Similar results were obtained in the C2-ceramide-stimulated cells in the absence of ionomycin. The level of intracellular free Ca^{2+} concentrations in PC12 cells was not modified by C2-ceramide at least in the period of 0–30 min after the addition (data not shown), as reported by Darios et al. (2003).

Table 1
Inhibition of [^{3}H]arachidonic acid release by D-*erythro*-sphingosine and its analogs, but not D-*erythro*-S1P, in PC12 cells

Addition	[^{3}H]Arachidonic acid release (% of total)	
	None	Ionomycin
None	0.80 ± 0.17	2.51 ± 0.24[a]
C2-Ceramide	2.44 ± 0.45[a]	5.47 ± 0.98[a,b]
D-*erythro*-Sphingosine	0.25 ± 0.14[a]	0.25 ± 0.25[b]
D-*erythro*-DMS	0.33 ± 0.09	1.39 ± 0.13[b]
D-*erythro*-DHS	0.23 ± 0.23	0.64 ± 0.18[b]
D-*erythro*-S1P	0.48 ± 0.17	3.14 ± 0.31
DL-*threo*-DHS	0.65 ± 0.12	2.55 ± 0.25

The prelabeled PC12 cells were detached from dishes and washed three times by centrifugation with the Tyrode-HEPES buffer. For measurement of [^{3}H]arachidonic acid release, cells were incubated for 30 min with vehicle or 100 μM of the indicated agents in the presence and absence of 5 μM ionomycin. Values are means ± S.E.M. for three to five independent experiments performed in triplicate.

[a] $P<0.05$, significantly different from the vehicle (none).

[b] $P<0.05$, significantly different from the value in the presence of 5 μ ionomycin.

Similar experiments were conducted in mouse fibroblast L929 cells, which were confirmed to express cPLA$_2\alpha$ (Hayakawa et al., 1993; Jayadev et al., 1997). The addition of 100 μM C2-ceramide also stimulated [^{3}H]arachidonic acid release from L929 cells with and without 5 μM ionomycin (Fig. 2). The addition of 50 μM D-*erythro*-sphingosine significantly inhibited basal and 100 μM C2-ceramide-stimulated [^{3}H]arachidonic acid releases from L929 cells.

Stimulation of cells with various agents that induce arachidonic acid release also promotes phosphorylation of cPLA$_2\alpha$ (Leslie, 1997; Hirabayashi and Shimizu, 2000). However, treatment with the inhibitors of protein kinases (20 μM U0126 for extracellular signal-regulated kinase (ERK) pathway, 10 μM SB203580 for p38 mitogen-activated protein kinase (MAPK), 10 μM Gö6976 for protein kinase C) did not inhibit the stimulatory effect of C2-ceramide in PC12 cells (data not shown). In addition,

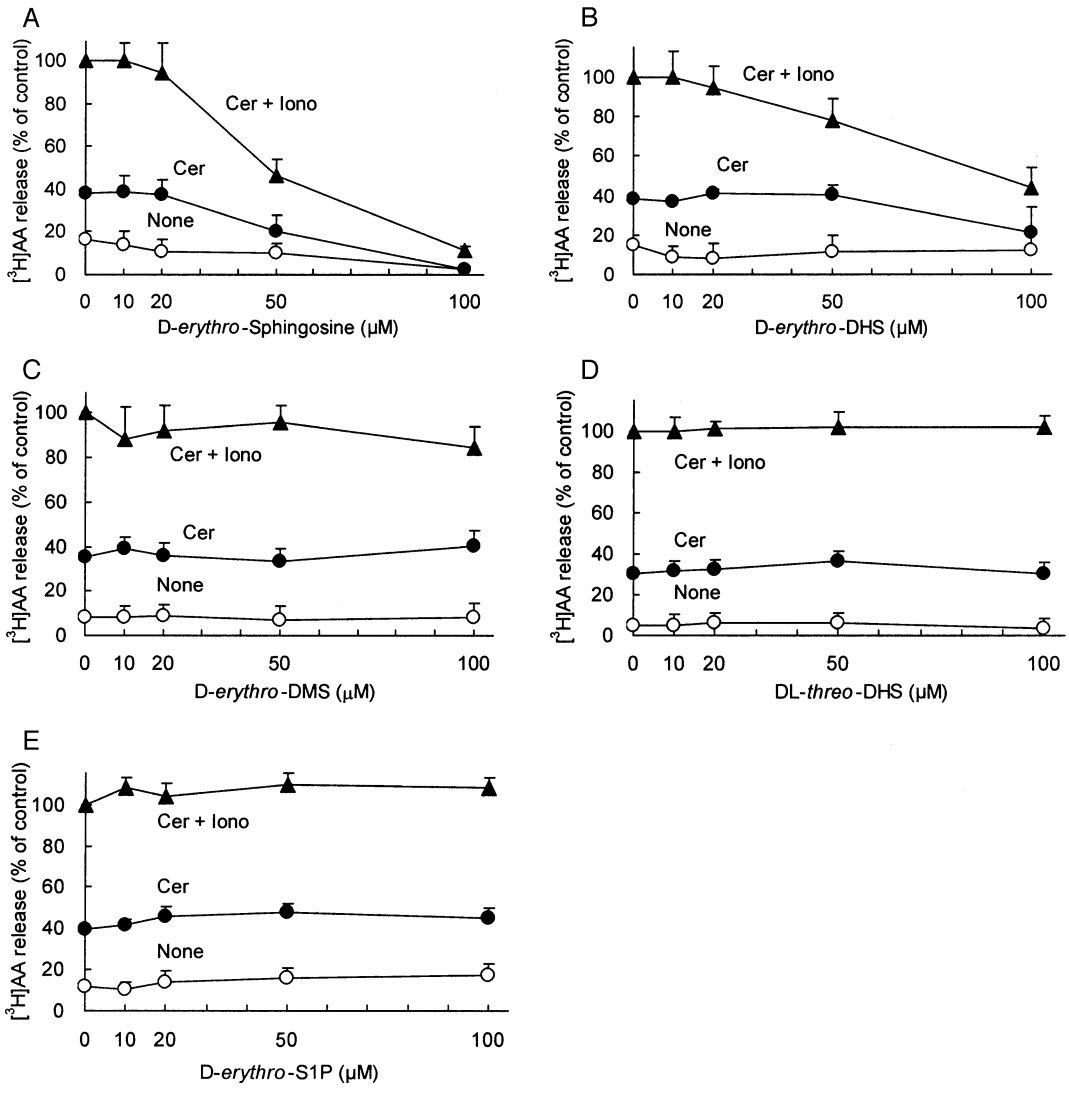

Fig. 1. Effects of D-*erythro*-sphingosine and its analogs on C2-ceramide-stimulated [^{3}H]arachidonic acid release in PC12 cells. The prelabeled PC12 cells were incubated for 30 min with the indicated concentrations of D-*erythro*-sphingosine (Panel A), D-*erythro*-DHS (Panel B), D-*erythro*-DMS (Panel C), DL-*threo*-DHS (Panel D) and D-*erythro*-S1P (Panel E) in the presence of vehicle (○), 100 μM C2-ceramide (Cer, ●) and 100 μM C2-ceramide plus 5 μM ionomycin (Iono, ▲). The assay mixture was supplemented with 2 mM $CaCl_2$. Values of [^{3}H]arachidonic acid release are normalized as percentages of the value induced by 100 μM C2-ceramide plus 5 μM ionomycin. The absolute values are shown in Table 1. Values are means ± S.E.M. for three independent experiments done in triplicate.

treatment with RHC80275 (100 μM, an inhibitor of diacylglycerol kinase) and bromoenol lactone (20 and 50 μM, a fairly selective inhibitor of Ca^{2+}-independent PLA_2) did not modify the C2-ceramide response (data not shown).

3.2. Effects of D-erythro-sphingosine analogs on mastoparan- and Na_3VO_4-stimulated [^{3}H]arachidonic acid release in PC12 cells

Previously, we reported that mastoparan (a wasp venom peptide) stimulated [^{3}H]arachidonic acid release from PC12 cells probably by activating $cPLA_2$ in the absence of extracellular $CaCl_2$ (Thang et al., 2000). Addition of D-*erythro*-sphingosine and D-*erythro*-DHS inhibited 20 μM mastoparan-stimulated [^{3}H]arachidonic acid release in the absence of $CaCl_2$ (Fig. 3). The inhibitory effects by these two analogs were detected at 10 μM and significant at concentrations greater than 20 μM. When used mastoparan as a stimulant, D-*erythro*-DMS inhibited the response in a concentration-dependent manner from 20 μM. D-*erythro*-S1P showed no effect at any concentrations and DL-*threo*-DHS at 100 μM showed a slight inhibitory effect (~ 30%) on mastoparan-stimulated [^{3}H]arachidonic acid release. The inhibitory effect of D-*erythro*-sphingosine on the C2-ceramide- and mastoparan-stimulated [^{3}H]arachidonic acid release was observed in the presence of 100 nM phorbol myristate acetate (an activator of conventional and novel types of protein kinase C, data not shown). Orthovanadate (Na_3VO_4), an inhibitor of tyrosine phosphatases, stimulated tyrosine phosphorylation in several proteins and arachidonic acid release in PC12 cells

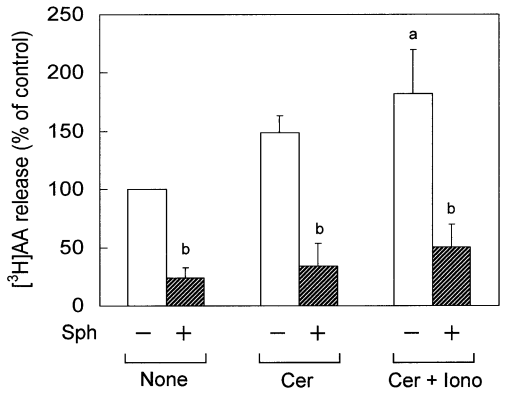

Fig. 2. Inhibition of C2-ceramide-stimulated [^{3}H]arachidonic acid release by D-*erythro*-sphingosine in L929 cells. The labeled L929 cells were incubated for 30 min in the presence of 2 mM $CaCl_2$ with vehicle or 100 μM C2-ceramide (Cer) with and without 5 μM ionomycin (Iono). The assay mixture was further supplemented with (hatched column) and without (while column) 50 μM D-*erythro*-sphingosine (Sph). Values of [^{3}H]arachidonic acid release are normalized as percentages of the basal release by vehicle (control). The absolute value of [^{3}H]arachidonic acid release was 5.83 ± 1.65 (% of total, $n = 3$). Values are means ± S.E.M. for three independent experiments done in triplicate. [a] $P < 0.05$, significantly different from the control value. [b] $P < 0.05$, significantly different from the value without D-*erythro*-sphingosine.

(Kitamura et al., 2000; Mori et al., 2001). In the presence of 5 and 10 mM Na_3VO_4, 100 μM C2-ceramide stimulated [^{3}H]arachidonic acid release (Table 2). Interestingly, neither 100 μM D-*erythro*-sphingosine nor 100 μM D-*erythro*-DMS inhibited Na_3VO_4-stimulated [^{3}H]arachidonic acid release.

Treatment with the tested sphingosine analogs including C2-ceramide at 100 μM for 1 h did not stimulate lactate dehydrogenase leakage from PC12 cells; for instance, the values are 5.7 ± 1.3%, 5.9 ± 2.1% and 5.4 ± 1.8% ($n = 3$) in the vehicle-, C2-ceramide-and D-*erythro*-sphingosine-treated cells, respectively, indicating that these reagents did not cause cell damage.

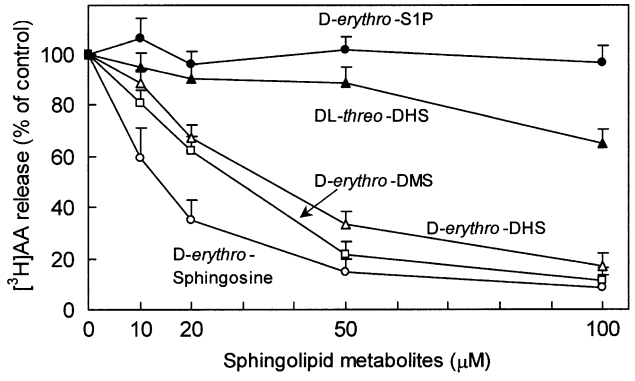

Fig. 3. Effects of D-*erythro*-sphingosine and its analogs on mastoparan-stimulated [^{3}H]arachidonic acid release in PC12 cells. The prelabeled PC12 cells were washed two times by centrifugation with $CaCl_2$-free buffer. For measurement of [^{3}H]arachidonic acid release, the cells were incubated for 30 min with 20 μM mastoparan in the absence of $CaCl_2$. The assay mixture was further supplemented with the indicated concentrations of D-*erythro*-sphingosine (○), D-*erythro*-DHS (△), D-*erythro*-DMS (□), DL-*threo*-DHS (▲) and D-*erythro*-S1P (●). The absolute value of [^{3}H]arachidonic acid release by 20 μM mastoparan was 8.51 ± 0.33 (% of total, $n = 5$). Values are means ± S.E.M. for three independent experiments done in triplicate.

Table 2
Effects of C2-ceramide, D-*erythro*-sphingosine and D-*erythro*-DMS on Na_3VO_4-stimulated [^{3}H]arachidonic acid release in PC12 cells

Additions	[^{3}H]Arachidonic acid release (% of total)		
	None	5 mM Na_3VO_4	10 mM Na_3VO_4
None	0.66 ± 0.10	3.83 ± 0.39[a]	7.27 ± 0.67[a]
C2-Ceramide	1.59 ± 0.26[a]	5.11 ± 0.29	11.53 ± 0.92
D-*erythro*-Sphingosine	0.29 ± 0.29	2.34 ± 0.35	8.93 ± 0.53
D-*erythro*-DMS	0.47 ± 0.13	4.54 ± 0.21	10.91 ± 1.24

The labeled PC12 cells were incubated for 30 min with the indicated agents (100 μM) in the presence of 2 mM $CaCl_2$. The assay mixture was further supplemented with vehicle or 5 and 10 mM Na_3VO_4. Values are means ± S.E.M. for three independent experiments performed in triplicate.
[a] $P < 0.05$, significantly different from the value without the agents.

3.3. Effects of pretreatment with D-erythro-DMS and D-erythro-sphingosine on arachidonic acid release in PC12 cells

The PC12 cells were treated with 100 μM D-*erythro*-DMS or 100 μM D-*erythro*-sphingosine for 20 min, and then washed twice by centrifugation in the buffer without these reagents (Table 3, Experiment I). [^{3}H]Arachidonic acid release induced by 20 μM mastoparan from the D-

Table 3
Effects of pretreatment with D-*erythro*-DMS and D-*erythro*-sphingosine on [^{3}H]arachidonic acid releases induced by C2-ceramide and mastoparan

Pretreatment	Control	D-*erythro*-DMS	D-*erythro*-Sphingosine
		[^{3}H]Arachidonic acid release (% of control)	
Experiment I (Pretreatment)			
None	100	134 ± 33	127 ± 33
C2-Ceramide + ionomycin	306 ± 46[a]	281 ± 31[a]	220 ± 18[a]
Mastoparan	1423 ± 191[a]	936 ± 220[a]	614 ± 131[a,b]
Experiment II (Pretreatment and assay mixture)			
None	100	100.4 ± 16.6	not determined
C2-Ceramide	240 ± 17[a]	748 ± 220[a,b]	not determined
Mastoparan	1555 ± 155	185 ± 25	not determined

The prelabeled PC12 cells were pretreated with vehicle, 100 μM D-*erythro*-DMS or 100 μM D-*erythro*-sphingosine for 20 min at 37 °C in the presence of 2 mM $CaCl_2$. The cells were washed with the buffer without the indicated sphingosine analogs two times, and then incubated for 30 min for measurement of [^{3}H]arachidonic acid release. In Experiment I, 100 μM C2-ceramide plus 5 μM ionomycin and 20 μM mastoparan were used as stimuli. In Experiment II, 300 μM C2-ceramide and 20 μM mastoparan were used as stimuli, and 100 μM D-*erythro*-DMS was supplemented in the assay mixture. Some values in Experiment II are means ± S.D. of two independent experiments ($n = 2$).
[a] $P < 0.05$, significantly different from the control value.
[b] $P < 0.05$, significantly different from the vehicle-treated cells.

erythro-DMS- and D-*erythro*-sphingosine-treated cells was much lower compared with that from the control cells. The release induced by 100 μM C2-ceramide plus 5 μM ionomycin from the D-*erythro*-DMS-treated cells was similar to that from the control cells, and the release from the D-*erythro*-sphingosine-treated cells was slightly inhibited compared with the control. When 100 μM D-*erythro*-DMS was supplemented with the assay mixture again, C2-ceramide-stimulated [^{3}H]arachidonic acid release was markedly enhanced in the D-*erythro*-DMS-treated cells, although the mastoparan response was inhibited in the treated cells (Experiment II).

3.4. Effects of C2-ceramide and D-erythro-sphingosine on prostaglandin $F_{2\alpha}$ formation in PC12 cells

Next we measured the content of prostaglandin $F_{2\alpha}$ in the medium of PC12 cells. The basal (non-stimulated) formation of prostaglandin $F_{2\alpha}$ for 30 min was 79.0 ± 21.2 pg/ml ($n = 4$), although the absolute value was variable depending on the experiments. The addition of 5 μM ionomycin significantly (about 2-fold) stimulated prostaglandin $F_{2\alpha}$ formation (Table 4). Addition of 20 μM C2-ceramide also stimulated prostaglandin $F_{2\alpha}$ formation (154.0 ± 7.5% of control, $n = 4$). Co-addition of 10 μM D-*erythro*-sphingosine significantly inhibited ionomycin-stimulated prostaglandin $F_{2\alpha}$ formation, without changing basal prostaglandin $F_{2\alpha}$ formation. The effects induced by C2-ceramide and D-*erythro*-sphingosine at higher concentrations were not determined because the agents alone slightly cross-reacted with the prostaglandin $F_{2\alpha}$ kit.

3.5. Effects of D-erythro-sphingosine analogs on cPLA$_2\alpha$ activity in vitro

To investigate the direct effects of sphingosine analogs on cPLA$_2\alpha$ enzyme, we measured cPLA$_2\alpha$ activity in vitro in the presence of these reagents (Fig. 4). We used the cytosol fractions of HEK293T cells transiently expressing human cPLA$_2\alpha$ for this purpose. The PLA$_2$ activity in 12.5 μg protein of HEK293T cells expressing cPLA$_2\alpha$ was 2000–4000 dpm/30 min depending on each experiment, whereas cells transfected with the control vector did not show detectable PLA$_2$ activity with under 50 dpm. In the absence of $CaCl_2$, the PLA$_2$ activities in the control and the transfected cells were very low (100 ~ 200 dpm). The addition of 20 μM D-*erythro*-sphingosine, D-*erythro*-DMS and D-*erythro*-DHS, but not D-*erythro*-S1P, markedly inhibited the cPLA$_2\alpha$ activity in the cytosol fractions from HEK293T cells transfected with vector encoding human cPLA$_2\alpha$. The addition of 20 μM DL-*threo*-DHS did not modify the activity, and C2-ceramide at 5 and 20 μM did not stimulate the activity in the present conditions (data not shown).

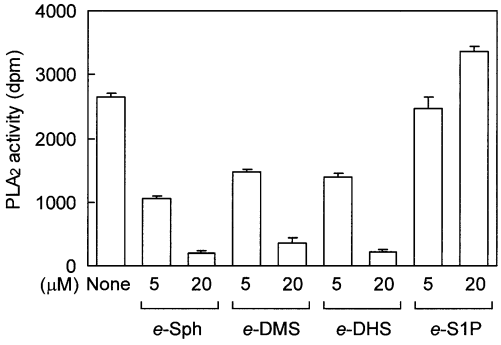

Fig. 4. Effects of D-*erythro*-sphingosine and its analogs on the cPLA$_2\alpha$ activity in vitro. The activity of cPLA$_2$ in the cytosol fractions from HEK293T cells expressing human cPLA$_2\alpha$ was measured as described in Materials and Methods. The assays were performed in the presence of vehicle or the indicated concentrations of D-*erythro*-sphingosine (*e*-Sph), D-*erythro*-DHS (*e*-DHS), D-*erythro*-DMS (*e*-DMS) or D-*erythro*-S1P (*e*-S1P). Values are means ± S.D. of three determinations in a typical experiment. The data are representative of three independent experiments.

Table 4
Inhibition of ionomycin-stimulated prostaglandin $F_{2\alpha}$ formation by D-*erythro*-sphingosine

	Vehicle	Net increase by ionomycin
	Prostaglandin $F_{2\alpha}$ formation (pg/ml)	
Vehicle	79.0 ± 21.2	70.0 ± 14.3[a]
Sphingosine	92.0 ± 15.1	19.3 ± 10.9[b]

PC12 cells were cultured with DME medium without fetal bovine serum in the presence of vehicle or 5 μM ionomycin for 30 min at 37 °C. The medium was further supplemented with 10 μM sphingosine. The amount of prostaglandin $F_{2\alpha}$ in the medium was measured using the EIA kit. Values are means ± S.E.M. for three to four independent experiments performed in triplicate.

[a] $P < 0.05$, significantly different from the control value.

[b] $P < 0.05$, significantly different from the value without sphingosine.

4. Discussion

4.1. C2-ceramide-stimulated arachidonic acid release and prostaglandin $F_{2\alpha}$ formation in PC12 cells

In the present study, we showed that the addition of C2-ceramide stimulated arachidonic acid release in PC12 cells and L929 cells, and prostaglandin $F_{2\alpha}$ formation in PC12 cells. The effect of C2-ceramide on [^{3}H]arachidonic acid release was marked in the presence of ionomycin and an inhibitor of Ca^{2+}-independent PLA$_2$ did not inhibit the C2-ceramide response. Addition of C2- and C6-ceramide stimulated arachidonic acid release from THP-1 monocytic cells (Pfau et al., 1998) and from mesangial cells (Huwiler et al., 2001), and C6-ceramide enhanced thrombin-stimulated arachidonic acid release and cPLA$_2$ activity in rabbit platelets (Sato et al., 1999). In addition, it has been reported C6-ceramide enhanced Ca^{2+}-ionophore-stimulated arachidonic acid release via translocation of cPLA$_2$ to membrane

fractions in platelets (Kitatani et al., 2000), and that ceramide bound to the Ca^{2+}-lipid binding domain of $cPLA_2$ and stimulated its activity in vitro (Huwiler et al., 2001). These findings suggest that C2-ceramide stimulates arachidonic acid release from cells probably via activation of $cPLA_2$ activity.

Activation of $cPLA_2\alpha$ required phosphorylation of serine residues by a member of MAPK family (Leslie, 1997; Hirabayashi and Shimizu, 2000), and the ERK pathway is involved in the ceramide-induced arachidonic acid release (Sato et al., 1999). In the present study, however, the inhibitor of the ERK pathway showed no effect on the release, as shown in mesangial cells (Huwiler et al., 2001). Another problem is that high concentrations of C2-ceramide greater than 100 μM were needed for arachidonic acid release in the tested cells. One of the reasons may that ceramide was metabolized to sphingosine and its derivatives in cells. As described below, some sphingosine analogs such as D-*erythro*-sphingosine and D-*erythro*-DHS inhibited arachidonic acid release in cells and/or $cPLA_2\alpha$ activity.

4.2. Inhibition of arachidonic acid release from cells and $cPLA_2\alpha$ activity by D-erythro-sphingosine and D-erythro-DHS, not D-erythro-S1P

D-*eryth ro*-sphingosine inhibited C2-ceramide- and/or ionomycin- and mastoparan-stimulated [^{3}H]arachidonic acid release in PC12 cells and L929 cells, and prostaglandin $F_{2\alpha}$ formation in PC12 cells. The inhibitory effect of D-*erythro*-sphingosine on the release appeared to be irreversible, because the mastoparan response from PC12 cells preincubated with D-*erythro*-sphingosine was significantly lower than that from the control cells. The addition of D-*erythro*-DHS to an assay mixture also inhibited C2-ceramide- and mastoparan-stimulated [^{3}H]arachidonic acid release. The inhibitory effects of D-*erythro*-sphingosine and D-*erythro*-DHS appeared to be selective, because neither D-*erythro*-S1P nor DL-*threo*-DHS showed the inhibitory effect. Since D-*erythro*-sphingosine and D-*erythro*-DMS did not inhibit the Na_3VO_4-stimulated release in PC12 cells, the effects of these molecules are not mediated through non-specific disruption of cell membranes. According to the results of LDH leakage assay, the stimulatory effect by C2-ceramide and the inhibitory effect by D-*erythro*-sphingosine on arachidonic acid release were not due to cell damage and/or cell toxicity. Sphingosine analogs such as D-*erythro*-sphingosine were inhibitors of protein kinase Cs (Lee et al., 1996), and sphingosine at concentrations greater than 20 μM inhibited arachidonic acid release via protein kinase C inhibition in platelets (Hashizume et al., 1997). However, the present results and those of a previous study (Murayama et al., 1995) suggest no involvement of the conventional and novel types of protein kinase Cs on the responses induced by C2-ceramide and D-*erythro*-sphingosine in PC12 cells. Since D-*erythro*-sphingosine and D-*erythro*-DHS inhibited the $cPLA_2\alpha$ activity *in vitro*, these sphingosine analogs appeared to inhibit PLA_2 activity directly in cells. The effectiveness of the sphingosine analogs on the Na_3VO_4-stimulated response may help to clarify the inhibitory mechanisms of sphingosine. A possible explanation is that the sphingosine analogs interact with the Ca^{2+}-lipid binding domain, not with the catalytic domains having the phosphorylation sites, of the $cPLA_2\alpha$ as described in Section 4.4.

PC12 cells express [EDG-5(H218)] and EDG-8 (Nrg-1) but not EDG-1 or -3 (Van Brocklyn et al., 1998, 1999). It was reported that exogenous addition of D-*erythro*-S1P caused cell rounding and neurite retraction via cell surface receptors, probably via EDG-5 (H218), in PC12 cells (Sato et al., 1997; Edsall et al., 1997; Van Brocklyn et al., 1998, 1999; MacLennan et al., 1999). Neither D-*erythro*-sphingosine, D-*erythro*-DHS nor D-*erythro*-DMS bound to EDG receptor family such as EDG-5 and -8 in these studies. D-*erythro*-Sphingosine and D-*erythro*-DHS are substrates for sphingosine kinases (Kohama et al., 1998), and exogenous addition of 10 μM sphingosine increased the intracellular S1P level by sphingosine kinases in PC12 cells (Sato et al., 1997). Thus, it is probable that the inhibitory effects of D-*erythro*-sphingosine and D-*erythro*-DHS on the responses (arachidonic acid release and $cPLA_2\alpha$ activity) are derived from D-*erythro*-S1P. However, exogenous addition of D-*erythro*-S1P showed no effect on the responses. D-*erythro*-DMS (an inhibitor of sphingosine kinases) showed inhibitory effects on the responses, similar to D-*erythro*-sphingosine. Thus, the inhibitory effects on arachidonic acid release (and probably prostaglandin $F_{2\alpha}$ formation) by D-*erythro*-sphingosine and D-*erythro*-DHS did not appear to be mediated by at least the reported EDG family receptors.

4.3. Effect of cellular metabolism of sphingosine analogs on arachidonic acid release

D-*erythro*-DMS showed dual (stimulatory and inhibitory) effects on [^{3}H]arachidonic acid release in PC12 cells. Addition of D-*erythro*-DMS to the assay mixture inhibited the ionomycin- and mastoparan-stimulated releases in PC12 cells, and inhibited the $cPLA_2\alpha$ activity in vitro. The inhibitory effect appeared to be irreversible at least partially. When C2-ceramide was used as a stimulant for arachidonic acid release, however, the effect of D-*erythro*-DMS by the simultaneous addition (Fig. 1B) or by the pretreatment (Table 3) was marginal. In contrast, pretreatment and co-addition of D-*erythro*-DMS enhanced C2-ceramide-stimulated [^{3}H]arachidonic acid release (Table 3, Experiment II). It was reported that treatment with D-*erythro*-DMS, an inhibitor of sphingosine kinases (Pyne and Pyne, 2000), decreased S1P levels but increased ceramide levels in PC12 cells (Edsall et al., 1998). Thus, D-*erythro*-DMS appeared to show two pharmacological effects in PC12 cells depending on conditions; (1) potentiation of the C2-ceramide response probably via inhibition of sphingosine kinases, and (2) inhibition of PLA_2 activity and thus inhibition of arachidonic acid release in cells. Ceramide-induced apopto-

sis of neuronal cells depended on conditions such as the cell density (Hartfield et al., 1997; Ping and Barrett, 1998), and the effects of sphingolipid metabolites on arachidonic acid metabolism are different in the cell types and the stimuli, as described in the Section 1. Recently, it is reported that ceramide kinase, via the formation of ceramide 1-phosphate, is an upstream modulator of PLA_2 activation (Pettus et al., 2003). These findings and reports suggest that a balance and the cellular metabolism of sphingolipid molecules in cells play a regulatory role on PLA_2 activity and/or arachidonic acid release. It should be determined the changes of sphingosine and/or ceramide levels in cells in response to stimuli in future.

4.4. Summary and problems

We showed that (1) C2-ceramide stimulated but D-*erythro*-sphingosine inhibited arachidonic acid release and prostaglandin $F_{2\alpha}$ formation in cells, (2) D-*erythro*-sphingosine , D-*erythro*-DHS and D-*erythro*-DMS, not D-*erythro*-S1P, inhibit arachidonic acid release, probably via inhibition of $cPLA_2\alpha$ activity. In our preliminary experiments, sphingosine inhibited the translocation of the $cPLA_2\alpha$ fused with a green fluorescent protein from the cytosol to the perinuclear region in response to Ca^{2+} in the transfected HEK293 cells (data not shown). It is reported that ceramide bound to the Ca^{2+}-lipid binding domain of $cPLA_2$ and accelerated the translocation of $cPLA_2$ to the membrane fractions (Kitatani et al., 2000; Huwiler et al., 2001). Determination of the precise mechanisms for stimulation by C2-ceramide and inhibition by sphingosine on arachidonic acid release remains to be solved. From the present results, we still could not exclude the possibility that sphingosine analogs and ceramide uptaken into the bilayers of membrane phospholipids modulated membrane susceptibility to PLA_2 (Franson et al., 1992; Klapisz et al., 2000).

References

Darios, F., Lambeng, N., Troadec, J.-D., Michel, P.P., Ruberg, M., 2003. Ceramide increases mitochondrial free calcium levels via caspase 8 and bid: role in initiation of cell death. J. Neurochem. 84, 643–654.

Edsall, L.C., Pirianov, G.G., Spiegel, S., 1997. Involvement of sphingosine 1-phosphate in nerve growth factor-mediated neuronal survival and differentiation. J. Neurosci. 17, 6952–6960.

Edsall, L.C., Van Brocklyn, J.R., Cuvillier, O., Kleuser, B., Spiegel, S., 1998. N,N-Dimethylsphingosine is a potent competitive inhibitor of sphingosine kinase but not of protein kinase C: modulation of cellular levels of sphingosine 1-phosphate and ceramide. Biochemistry 37, 12892–12898.

Edsall, L.C., Cuvillier, O., Twitty, S., Spiegel, S., Milstien, S., 2001. Sphingosine kinase expression regulates apoptosis and caspase activation in PC12 cells. J. Neurochem. 76, 1573–1584.

Franson, R.C., Harris, L.K., Ghosh, S.S., Rosenthal, M.D., 1992. Sphingolipid metabolism and signal transduction: inhibition of in vitro phospholipase activity by sphingosine. Biochim. Biophys. Acta 1136, 169–174.

Hannun, Y., 1996. Functions of ceramide in coordinating cellular responses to stress. Science 274, 1855–1859.

Hartfield, P.J., Mayne, G.C., Murray, A.W., 1997. Ceramide induces apoptosis in PC12 cells. FEBS Lett. 401, 148–152.

Hashizume, T., Nakao, M., Kageura, T., Sato, T., 1997. Sphingosine enhances arachidonic acid liberation in response to U46619 through an increase in phospholipase A_2 activity in rabbit platelets. J. Biochem. (Tokyo) 122, 1034–1039.

Hashizume, T., Kitatani, K., Kageura, T., Hayama, M., Akiba, S., Sato, T., 1999. Ceramide enhances susceptibility of membrane phospholipids to phospholipase A_2 through modification of lipid organization in platelet membranes. Biol. Pharm. Bull. 22, 1275–1278.

Hayakawa, M., Ishida, N., Takeuchi, K., Shibamoto, S., Hori, T., Oku, N., Ito, F., Tsujimoto, M., 1993. Arachidonic acid-selective cytosolic phospholipase A_2 is crucial in the cytotoxic action of tumor necrosis factor. J. Biol. Chem. 268, 11290–11295.

Hirabayashi, T., Shimizu, T., 2000. Localization and regulation of cytosolic phospholipase A_2. Biochim. Biophys. Acta 1488, 124–138.

Huwiler, A., Johansen, B., Skarstad, A., Pfeilschifter, J., 2001. Ceramide binds to the CaLB domain of cytosolic phospholipase A_2 and facilitates its membrane docking and arachidonic acid release. FASEB J. 15, 7–9.

Igarashi, Y., 1997. Functional roles of sphingosine, sphingosine 1-phosphate, and methylsphingosine: in regard to membrane sphingolipid signaling pathways. J. Biochem. (Tokyo). 122, 1080–1087.

Jayadev, S., Hayter, H.L., Andrieu, N., Gamard, C.J., Liu, B., Balu, R., Hayakawa, M., Ito, F., Hannun, Y.A., 1997. Phospholipase A_2 is necessary for tumor necrosis factor α-induced ceramide generation in L929 cells. J. Biol. Chem. 272, 17196–17203.

Kitamura, T., Murayama, T., Nomura, Y., 2000. Enhancement of Ca^{2+}-induced noradrenaline release by vanadate in PC12 cells: possible involvement of tyrosine phospholylation. Brain Res. 854, 165–171.

Kitatani, K., Oka, T., Murata, T., Hayama, M., Akiba, S., Sato, T., 2000. Acceleration by ceramide of calcium-dependent translocation of phospholipase A_2 from cytosol to membranes in platelets. Arch. Biochem. Biophys. 382, 296–302.

Kitatani, K., Akiba, S., Hayama, M., Sato, T., 2001. Ceramide accelerates dephosphorylation of extracellular signal-regulated kinase 1/2 to decrease prostaglandin D_2 production in RBL-2H3 cells. Arch. Biochem. Biophys. 395, 208–214.

Klapisz, E., Masliah, J., Béréziat, G., Wolf, C., Koumanov, K.S., 2000. Sphingolipids and cholesterol modulate membrane susceptibility to cytosolic phospholipase A_2. J. Lipid Res. 41, 1680–1688.

Kohama, T., Olivera, A., Edsall, L., Nagiec, M.M., Dickson, R., Spiegel, S., 1998. Molecular cloning and functional characterization of murine sphingosine kinase. J. Biol. Chem. 273, 23722–23728.

Lee, J.Y., Hannun, Y.A., Obeid, L.M., 1996. Ceramide inactivates cellular protein kinase Cα. J. Biol. Chem. 271, 13169–13174.

Leslie, C.C., 1997. Properties and regulation of cytosolic phospholipase A_2. J. Biol. Chem. 272, 16709–16712.

Levade, T., Jaffrézou, J.-P., 1999. Signalling sphingomyelinases: which, where, how and why? Biochim. Biophys. Acta 1438, 1–17.

MacLennan, A.J., Devlin, B.K., Marks, L., Gaskin, A.A., Neitzel, K.L., Lee, N., 1999. Antisense studies in PC12 cells suggest a role for H218, a sphingosine 1-phosphate receptor, in growth-factor-induced cell-cell interaction and neurite outgrowth. Dev. Neurosci. 22, 283–295.

Mori, A., Yasuda, Y., Murayama, T., Nomura, Y., 2001. Enhancement of arachidonic acid release and prostaglandin $F_{2\alpha}$ formation by Na3VO4 in PC12 cells and GH3 cells. Eur. J. Pharmacol. 417, 19–25.

Murayama, T., Oda, H., Watanabe, A., Nomura, Y., 1995. ATP receptor-mediated increase of Ca ionophore-stimulated arachidonic acid release from PC12 pheochromocytoma cells. Jpn. J. Pharmacol. 69, 43–51.

Muthalif, M.M., Benter, I.F., Uddin, M.R., Malik, K.I., 1996. Calcium/calmodulin-dependent protein kinase IIα mediates activation of mitogen-activated protein kinase and cytosolic phospholipase A_2 in norepinephrine-induced arachidonic acid release in rabbit aortic smooth muscle cells. J. Biol. Chem. 271, 30149–30157.

Olivera, A., Kohama, T., Edsall, L., Nava, V., Cuvillier, O., Poulton, S., Spiegel, S., 1999. Sphingosine kinase expression increases intracellular

sphingosine-1-phosphate and promotes cell growth and survival. J. Cell Biol. 147, 545–557.

Pettus, B.J., Bielawska, A., Spiegel, S., Roddy, P., Hannun, Y.A., Chalfant, C.E., 2003. Ceramide kinase mediates cytokine- and calcium ionophore-induced arachidonic acid release. J. Biol. Chem. 278, 38206–38213.

Pfau, J.C., Walker, E.B., Card, G.L., 1998. A comparison of the effects of lipopolysaccharide and ceramide on arachidonic acid metabolism in THP-1 monocytic cells. Cell. Immunol. 186, 147–153.

Ping, S.E., Barrett, G.L., 1998. Ceramide can induced cell death in sensory neurons, whereas ceramide analogs and sphingosine promote survival. J. Neurosci. Res. 54, 206–213.

Pyne, S., Pyne, N.J., 2000. Sphingosine 1-phosphate signalling in mammalian cells. Biochem. J. 349, 385–402.

Sato, K., Tomura, H., Igarashi, Y., Ui, M., Okajima, F., 1997. Exogenous sphingosine 1-phosphate induces neurite retraction possibly through a cell surface receptor in PC12 cells. Biochem. Biophys. Res. Commun. 240, 329–334.

Sato, T., Kageura, T., Hashizume, H., Hayama, M., Kitatani, K., Akiba, S., 1999. Stimulation by ceramide of phospholipase A_2 activation through a mechanism related to the phospholipase C-initiated signaling pathway in rabbit platelets. J. Biochem. (Tokyo) 125, 96–102.

Someya, A., Horie, S., Murayama, T., 2002. Arachidonic acid release and prostaglandin $F_{2\alpha}$ formation induced by anandamide and capsaicin in PC12 cells. Eur. J. Pharmacol. 450, 131–139.

Spiegel, S., 1999. Sphingosine 1-phosphate: a prototype of a new class of second messengers. J. Leukoc. Biol. 65, 341–344.

Surette, M.E., Winkler, J.D., Fonteh, A.N., Chilton, F.H., 1996. Relationship between arachidonate-phospholipid remodeling and apoptosis. Biochemistry 35, 9187–9196.

Thang, S.H., Yasuda, Y., Umezawa, M., Murayama, T., Nomuya, Y., 2000. Inhibition of phospholipase A_2 activity by *S*-nitroso-cysteine in a cyclic GMP-independent manner in PC12 cells. Eur. J. Pharmacol. 395, 183–191.

Van Brocklyn, J.R., Lee, M.-J., Menzeleev, R., Olivera, A., Edsall, L., Cuvillier, O., Thomas, D.M., Coopman, P.J.P., Thangada, S., Liu, C.H., Hla, T., Spiegel, S., 1998. Dual actions of sphingosine-1-phosphate: extracellular through the G_i-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. J. Cell Biol. 142, 229–240.

Van Brocklyn, J.R., Tu, Z., Edsall, L.C., Schmidt, R.R., Spiegel, S., 1999. Sphingosine 1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218. J. Biol. Chem. 274, 4626–4632.

Wiegmann, K., Schütze, S., Machleidt, T., Witte, D., Krönke, M., 1994. Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. Cell 78, 1005–1015.

Yasuda, Y., Yoshinaga, N., Murayama, T., Nomura, Y., 1999. Inhibition of hydrogen peroxide-induced apoptosis but not arachidonic acid release in GH3 cells by EGF. Brain Res. 850, 197–206.

Yoshimura, S., Banno, Y., Nakashima, S., Takenaka, K., Sakai, H., Nishimura, Y., Sakai, N., Shimizu, S., Eguchi, Y., Tsujimoto, Y., Nozawa, Y., 1998. Ceramide formation leads to caspase-3 activation during hypoxic PC12 cell death. J. Biol. Chem. 273, 6921–6927.