Stimulation of DNA synthesis by natural ceramide 1-phosphate

Antonio GOMEZ-MUÑOZ*†, Laura M. FRAGO*, Luis ALVAREZ and Isabel VARELA-NIETO

Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas (CSIC), C/Arturo Duperier 4, 28029-Madrid, Spain (Instituto Asociado al Grupo de Biomembranas de la Facultad de Ciencias de la Universidad del País Vasco, P.O. Box 644, Bilbao, Spain)

We found that natural (long-chain) ceramide 1-phosphate can be dispersed into aqueous solution when dissolved in an appropriate mixture of methanol/dodecane (49:1, v/v). This solvent mixture facilitates the interaction of this phosphosphingolipid with cells. Under these conditions, incubation of EGFR T17 fibroblasts with natural ceramide 1-phosphate caused a potent stimulation of DNA synthesis. This effect was accompanied by an increase in the levels of proliferating-cell nuclear antigen. Concentrations of natural ceramide 1-phosphate that stimulated the synthesis of DNA did not inhibit adenylate cyclase activity, nor did they stimulate phospholipase D. Natural ceramide 1-phosphate did not alter the cellular phosphorylation state of tyrosine residues or of mitogen-activated protein kinase. Furthermore, natural ceramide 1-phosphate failed to induce the expression of the proto-oncogenes c-*myc* and c-*fos*. Both the stimulation of DNA synthesis and the induction of proliferating-cell nuclear antigen by natural ceramide 1-phosphate were inhibited by natural ceramides. This work suggests that the use of methanol and dodecane to deliver natural ceramide 1-phosphate to cells may be useful for elucidation of the biological function(s) and mechanism(s) of action of ceramide 1-phosphate.

INTRODUCTION

The agonist-stimulated breakdown of sphingomyelin plays an important role in signal transduction [1–3]. Stimulation of cells with 1,25-dihydroxyvitamin D_3 [4], tumour necrosis factor-α, interleukin-1 or γ-interferon [1–3] activates a neutral sphingomyelinase at the plasma membrane of cells, generating intracellular ceramides and phosphocholine. In addition, ceramides can be formed through the action of an acidic sphingomyelinase that is activated by 1,2-diacylglycerol [1–3]. Ceramides are now recognized to be important second messengers. They can cause: (a) induction of anti-proliferative effects in human leukaemia HL-60 cells [4,5] and in rat fibroblasts stimulated with phosphatidate (PA), lysoPA [6] or sphingosine 1-phosphate (SPP) [7]; (b) a decrease in the mRNA for the proto-oncogene c-*myc* in HL-60 cells [8]; (c) stimulation of protein kinase [9] and phosphatase [10] activities; (d) phosphorylation of the epidermal growth factor (EGF) receptor in A431 human epidermal carcinoma cells [11]; (e) induction of programmed cell death (apoptosis) in different cell types [12]; and (f) up-regulation of the mRNA levels of cytosolic phospholipase A_2 and a mitogen-inducible form of cyclo-oxygenase [13]. Exogenous sphingomyelinase and cell-permeable ceramides also induce mitogen-activated protein kinase (MAP kinase) activation in HL-60 cells [14]; block the activation of phospholipase D (PLD) by various agonists, including serum, thrombin, lysoPA and SPP [6,7]; stimulate a stress-activated protein kinase [15]; and decrease the insulin-induced tyrosine phosphorylation of insulin receptor substrate-1 [16].

Interestingly, a novel pathway has been described in which ceramide originating from the action of neutral sphingomyelinase, but not glucosylceramidase, is converted into ceramide 1-phosphate (Cer1*P*) by a Ca^{2+}-dependent kinase [17]. Cer1*P* has been detected in human leukaemia HL-60 cells [17] and brain tissue [18]. Shinghal et al. [18] identified a Cer1*P* phosphatase in rat brain, suggesting that Cer1*P* might regulate some aspects of synaptic vesicle functioning, and Boudker and Futerman [19] characterized a plasma membrane phosphatase that specifically hydrolyses Cer1*P*. Furthermore, Cer1*P* can be converted into ceramide by the action of a phosphatidate phosphohydrolase that is specifically located in the plasma membranes of cells [20]. These results indicate that Cer1*P* can be turned over and that it may play an important role in cell activation. However, little is known about the physiological effects of this phospholipid. It was demonstrated recently [21] that synthetic short-chain Cer1*P* can stimulate the synthesis of DNA and cell division when presented in a sonicated form in water to subconfluent cultures of rat fibroblasts. However, natural Cer1*P* was without effect under the same conditions.

In the present study we have developed a solvent system for the efficient delivery of natural Cer1*P* that allows interaction of the phospholipid with cells in culture. We show here for the first time that natural Cer1*P*, dispersed in methanol/dodecane (49:1, v/v), stimulates the synthesis of DNA and the expression of proliferating-cell nuclear antigen (PCNA). The latter is a non-histone nuclear protein that is essential for cell cycle progression [22]. We also show that the effects of natural Cer1*P* can be blocked by natural ceramides. This work therefore identifies a biological action of natural Cer1*P* as a putative mitogenic agent. The use of an appropriate mixture of methanol and dodecane to deliver natural Cer1*P* to cells will probably shed light on the mechanism by which this phospholipid exerts its physiological effects.

MATERIALS AND METHODS

Materials

Anti-phosphotyrosine antibodies (4G10) were from UBI (Lake

Abbreviations used: C_2, acetyl; C_8, octanoyl; Cer1*P*, ceramide 1-phosphate; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; MAP kinase, mitogen-activated protein kinase; PA, phosphatidate; PCNA, proliferating-cell nuclear antigen; PLD, phospholipase D; SPP, sphingosine 1-phosphate.

* These authors contributed equally to the present work.

† To whom correspondence should be addressed.

Placid, NY, U.S.A.). Antibodies to MAP kinase were from Zymed Laboratories (San Francisco, CA, U.S.A.). Antibodies to phosphorylated MAP kinase were from Biolabs (Beverly, MA, U.S.A.), and antibodies to PCNA were from Concepta Biosystems SA (Barcelona, Spain). Dulbecco's modified Eagle's medium (DMEM) was from Biowhittaker (Verviers, Belgium). Ceramide (type III), cardiolipin (from bovine heart), n-octyl β-D-glucopyranoside, dithiothreitol, diethylenetriaminepenta-acetic acid and sphingomyelinase (from *Staphylococcus aureus*) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetyl (C_2)-and octanoyl (C_8)-ceramides were from Matreya (Pleasant Gap, PA, U.S.A.). [^{3}H]cAMP radioimmunoassays, Readyprime labelling kits and [^{3}H]thymidine (49 Ci/mmol) were from Amersham (Little Chalfont, Bucks, U.K.). Diacylglycerol kinase was from Calbiochem (San Diego, CA, U.S.A.). Nytran and PVDF membranes were from Schleicher and Schuell (Keene, NH, U.S.A.). Pluronic was from Molecular Probes (Eugene, OR, U.S.A.).

Culture of fibroblasts

EGFR T17 cells (which are NIH 3T3 fibroblasts overexpressing the human EGF receptor [23]) were maintained in DMEM supplemented with 10 % (v/v) foetal bovine serum, 10 units/ml penicillin and 10 μg/ml streptomycin. Fibroblasts were seeded at 3×10^4 cells/well on 24-well culture dishes and incubated at 37 °C in 5 % CO_2 in air for 3 days, except for determinations of PLD activity, for which 35 mm culture dishes were used with equivalent cell densities. Where indicated, the fibroblasts were treated with dispersions of ceramide or Cer1*P* that were prepared in solvent mixtures of ethanol or methanol/dodecane (49:1, v/v), as indicated. The final concentration of solvent in the medium was about 3.3 %. Control incubations of fibroblasts were treated with equal volumes of solvent in the absence of lipid for comparison.

Synthesis of Cer1*P*

Cer1*P* was prepared enzymically as described by Schneider and Kennedy [24] and adapted by Preiss et al. [25], with modifications [21]. Briefly, type III ceramide or C_8-ceramide (2 mg) was solubilized by sonication in 5 mM cardiolipin, 7.5 % octyl β-glucopyranoside and 1 mM diethylenetriaminepenta-acetic acid, and then resuspended in a mixture containing 50 mM imidazole, pH 6.6, 50 mM NaCl, 100 mM $MgCl_2$, 1 mM EGTA and 0.4 unit/ml diacylglycerol kinase. The reaction was started with 25 mM ATP. After 12 h at 37 °C the incubation was supplemented with 0.4 unit/ml diacylglycerol kinase and the reaction was continued for a further 2 h. The reaction was stopped by extraction of the lipids as previously described [7,21]. The organic phase was dried under N_2 and the lipids analysed as described in [7,21]. The lipid was applied to thin-layer plates in chloroform/methanol (1:1, v/v). The plates were developed sequentially with chloroform/methanol/NH_4OH (65:35:7.5, by vol.) and chloroform/acetone/acetic acid/methanol/water (10:4:3:2:1, by vol.). To identify Cer1*P* on the thin-layer plate, [γ-^{32}P]ATP was included in some of the reaction tubes. [^{32}P]Cer1*P* was identified as a single spot at R_F 0.51. Radioactive and unlabelled Cer1*P* was eluted with three washes of 3 ml of chloroform/methanol/acetic acid/water (50:39:1:1, by vol.). Silica was removed by centrifugation and filtration, and 1 ml of water was added to the combined supernatants to separate the phases. The organic phase was washed once with 1 ml of methanol/water (1:1, v/v). Cer1*P* was standardized by phosphate analysis [17] and was stored in chloroform/methanol (1:1, v/v) at −20 °C.

To characterize Cer1*P* further, it was deacylated in 6 M HCl/butan-1-ol (1:1, v/v) for 60 min at 100 °C, and SPP was resolved by TLC using butan-1-ol/acetic acid/water (3:1:1, by vol.). Recovery of Cer1*P* as SPP was about 65 %, as previously reported [17,21]. SPP was identified using sprays specific for phosphate and amino groups, and by using an authentic standard that was prepared as described in [7]. The purity of Cer1*P* was confirmed by TLC using two additional solvent systems. System I consisted of chloroform/methanol/acetic acid (65:15:5, by vol.), and Cer1*P* was identified by autoradiography as a single spot at R_F 0.26. System II consisted of three diferent solvent mixtures: A, chloroform/methanol/NH_4OH (65:35:7.5, by vol.); B, chloroform/methanol/acetic acid (9:1:1, by vol.); C, butan-1-ol/acetic acid/water (3:1:1, by vol.). Samples were run sequentially in one dimension as indicated [7]. The positions of non-phosphate-containing sphingolipids could be identified by staining with 0.2 % 2′,7′-dichlorofluorescein in 95 % ethanol and viewing the lipid spots under light at 366 nm. Cer1*P* was identified as a single spot at R_F 0.50 by autoradiography or by spraying the plates with Molybdenum Blue reagent [7].

Determination of DNA synthesis

Fibroblasts were grown as indicated above and were maintained in medium containing 0.1 % foetal bovine serum for 24 h to cause growth arrest. Cells were then incubated in the presence or absence of agonists as indicated for 24 h in serum-free medium, and [^{3}H]thymidine (0.5 μCi/dish) was added for the last 6 h of the incubation. The cells were washed twice with PBS and the incorporation of [^{3}H]thymidine into DNA was measured as described previously [6,7,21].

Assay of cAMP

For these measurements, the lysates from the cells were boiled for 5 min and centrifuged at 12000 ***g*** for 10 min. Samples of the supernatants were then used to determine cAMP concentrations by using a radioimmunoassay kit from Amersham [26].

Assay of PLD activity and ceramide production

The activity of PLD was determined by measuring the accumulation of [^{3}H]phosphatidylethanol, which is the product of its transphosphatidylation reaction and is considered to be a definitive assay for PLD [27]. The labelling of fibroblasts with [^{3}H]myristate and the details of the PLD assay have been described previously [28]. The formation of [^{3}H]ceramide was determined by scraping the ceramide from the same thin-layer plate as that used for isolating the [^{3}H]phosphatidylethanol. The identity of the ceramide was confirmed by co-chromatography with authentic natural ceramide. Similar studies on ceramide formation were also performed after labelling fibroblasts with [^{3}H]palmitate under the same conditions [7].

Incorporation of [^{32}P]Cer1*P* into EGFR T17 fibroblasts

Cells were preincubated in DMEM containing 0.1 % BSA for 2 h. [^{32}P]Cer1*P* (25 μM; 0.44 Ci/mol), which was sonicated in water or dispersed in methanol/dodecane (49:1, v/v), was then added to the cell cultures for the times indicated. The medium was removed and cells were washed once with DMEM containing 0.1 % BSA, and twice with ice-cold PBS. The cells were then scraped into 0.5 ml of methanol and the dishes washed with an additional 0.5 ml of methanol. Chloroform (0.5 ml) was then added to the combined methanol samples. Lipids were extracted

by separation of the phases with an additional 0.7 ml of chloroform, 0.2 ml of methanol and 0.9 ml of 2 M KCl containing 0.2 M H_3PO_4. Lipids were separated on glass-backed silica-gel G thin-layer plates, as previously reported [21]. Radioactive Cer1*P* was identified by autoradiography and quantified, after being scraped from the plates, by liquid scintillation counting.

Western blot analysis of tyrosine and MAP kinase phosphorylation, and of PCNA

Fibroblasts were incubated for 24 h in DMEM containing 0.1 % foetal calf serum and then treated with agonists in the absence of serum for 5 min. Cells were lysed on the dishes by addition of buffer containing 10 mM Tris/HCl, pH 7.2, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 1 % sodium deoxycholate, 5 mM EDTA, 20 mM NaF, 1 mM vanadate, 50 μg/ml leupeptin, 50 μg/ml aprotinin and 1 mM PMSF. Proteins were measured by a colorimetric microassay (Micro BCA; Pierce, Rockford, IL, U.S.A.). Cell lysates were centrifuged at 12000 ***g*** for 15 min at 4 °C. Aliquots of the supernatants were normalized for protein, mixed with concentrated (5 ×) Laemmli sample buffer, boiled for 5 min and then resolved by SDS/PAGE on 7.5 %, 8 %, 10 % and 10 % acrylamide/bisacrylamide gels for determination of phosphotyrosine, MAP kinase, phospho-MAP kinase and PCNA respectively, under reducing conditions. Proteins were then transferred to a PVDF membrane. Filters were blocked with Tris-buffered saline containing 3 % BSA for phosphotyrosine determinations, or with 5 % (w/v) non-fat dried milk for MAP kinase, phospho-MAP kinase and PCNA determinations, and then incubated overnight. Incubations were continued for 3 h with anti-phosphotyrosine, anti-(MAP kinase), anti-(phospho-MAP kinase) and anti-PCNA mouse monoclonal antibodies, which were diluted 1:40, 1:3000, 1:1000 and 1:3000 respectively. Filters were then washed and incubated with a 1:2500 dilution of goat anti-mouse immunoglobulin conjugated to horseradish peroxidase. Bound peroxidase activity was visualized by chemiluminiscence (Rennaisance; Dupont-NEN, Wilmington, DE, U.S.A.).

Measurement of intracellular calcium levels

The intracellular Ca^{2+} concentration was determined by using the fluorescent probe fura-2 [29], as described by Junco et al. [30]. Briefly, EGFR T17 fibroblasts were grown on glass coverslips and loaded with fura-2 acetoxymethyl ester (5 μM) for 30 min at 37 °C in DMEM supplemented with Pluronic (100 μM) and 0.1 % BSA. Subsequently, cells were washed three times with PBS and agonists were added after establishment of a steady baseline for intracellular Ca^{2+} levels.

Northern blot analysis

EGFR T17 cells were grown to 70–80 % confluence in DMEM supplemented with 10 % (v/v) foetal bovine serum. Cells were then changed to medium containing 0.1 % foetal bovine serum 24 h before the addition of the indicated agonists. Total RNA was isolated by the guanidinium isothiocyanate method [31], fractionated on a 1 % agarose denaturing gel, transferred to Nytran membranes and hybridized to radiolabelled c-*fos* [32] or c-*myc* [33] cDNA as reported [34]. The probes were random-prime-labelled with [α-^{32}P]dCTP using a Readyprime labelling kit from Amersham. The filters were scanned on a Molecular Imager GS-250 (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Presentation of results

All data are expressed as means ± S.E.M. unless stated otherwise. Statistical evaluation was by unpaired Student's *t* test.

RESULTS

It has been shown previously that short-chain Cer1*P* can be used as an external agonist to stimulate cell proliferation [21]. Exogenous natural (long-chain) Cer1*P* was not effective in inducing the synthesis of DNA under the same conditions, and therefore the effects of the synthetic short-chain Cer1*P* were considered to be pharmacological in nature [21]. In the present study, we show for the first time that natural Cer1*P* stimulates the synthesis of DNA ($P < 0.03$) potently, when presented to subconfluent cultures of EGFR T17 fibroblasts in a solvent mixture of methanol/dodecane (49:1, v/v), after 24 h of incubation (Figure 1). We also demonstrate that both natural Cer1*P* and C_8-Cer1*P* are able to increase ($P < 0.03$) the levels of PCNA in the fibroblasts after 24 h of incubation (Figure 2 and Table 1). We

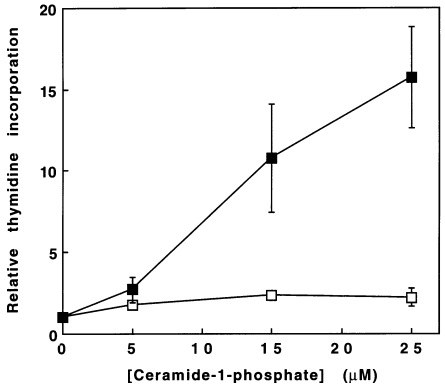

Figure 1 Stimulation of DNA synthesis by natural Cer1*P*

EGFR T17 fibroblasts were incubated for 24 h in serum-free DMEM with methanol/dodecane (49:1, v/v) alone (□) or with natural Cer1*P* (■) at the concentrations indicated. [^{3}H]Thymidine was present during the last 6 h of the incubation. Results are expressed relative to the control value in the absence of methanol/dodecane and phospholipid, which is the 0 μM Cer1*P* concentration point. The values are given as means ± range of two independent experiments performed in triplicate, except for the point at 25 μM where the values are means ± S.E.M. of five independent experiments. The significance of the difference between incubations in the absence and presence of 25 μM Cer1*P* has a value of $P < 0.03$. Basal [^{3}H]thymidine incorporation corresponded to 5600 ± 1786 d.p.m./dish in seven independent experiments.

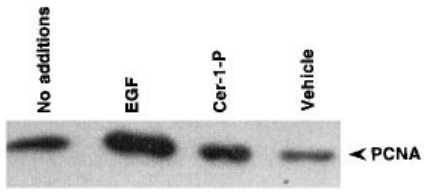

Figure 2 Stimulation of PCNA expression by Cer1*P*

EGFR T17 fibroblasts were incubated for 24 h in serum-free DMEM without or with methanol/dodecane (49:1, v/v) alone (vehicle), natural Cer1*P* (25 μM) or EGF (10 nM) as indicated. Lysates (30 μg of protein) of the fibroblasts were analysed by SDS/PAGE and Western blotting, performed as described in the Material and methods section.

Table 1 Ceramides counteract the stimulation of PCNA expression by Cer1*P*

EGFR T17 fibroblasts were incubated for 24 h in serum-free DMEM in the absence or presence of methanol/dodecane (49:1, v/v) (vehicle), EGF (10 nM), natural Cer1*P* (25 μM), C_8-Cer1*P* (5 μM), natural ceramides (10 μM) or C_2-ceramide (10 μM), as indicated. Lysates (30 μg of protein) from fibroblasts were analysed by SDS/PAGE, and Western blotting was performed as described in the Materials and methods section. PCNA spots were quantified by densitometry. Results are expressed relative to the control value, and are means $\pm$ S.E.M. of five independent experiments performed in duplicate. Significance of differences: $^*P < 0.03$ between incubations in the absence and the presence of natural Cer1*P* or C_8-Cer1*P*; †$P < 0.02$ between incubations with natural Cer1*P* in the absence and the presence of 10 μM natural ceramides, or with C_8-Cer1*P* in the absence and the presence of 10 μM C_2-ceramide.

Addition	PCNA levels (fold increase)
None	1.0
Vehicle	1.04 $\pm$ 0.15
EGF	2.65 $\pm$ 0.43
Natural Cer1*P*	1.78 $\pm$ 0.24*
C_8-Cer1*P*	1.76 $\pm$ 0.22*
Natural Cer1*P* + natural ceramides	0.91 $\pm$ 0.21†
C_8-Cer1*P* + C_2-ceramide	0.94 $\pm$ 0.25†

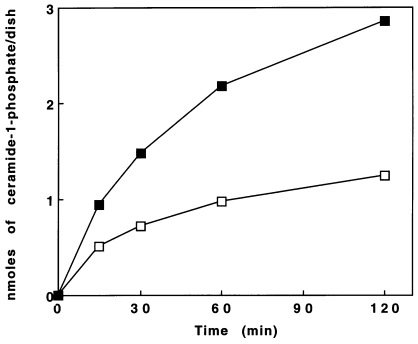

Figure 3 Incorporation of Cer1*P* into fibroblasts

EGFR T17 fibroblasts were incubated in serum-free DMEM containing 0.5% BSA for the indicated periods of time with 25 μM [^{32}P]Cer1*P* (0.44 Ci/mol), which was delivered in water (□) or in methanol/dodecane (49:1, v/v) (■). Lipids were analysed as described in the Materials and methods section. Results are from a representative experiment performed in triplicate, and were confirmed in a second experiment with an extra time point at 5 h. The incorporation of [^{32}P]Cer1*P* after 5 h of incubation was about 2-fold greater when the radiolabelled phospholipid was presented to the cells in methanol/dodecane compared with water.

found that sonication of natural Cer1*P* in methanol/dodecane (49:1, v/v) helps dispersion of this phospholipid in aqueous solution and facilitates its interaction with the cells. Figure 3 shows that the cellular uptake of [^{32}P]Cer1*P* by the fibroblasts was considerably greater when the radiolabelled phospholipid was added to the cells in methanol/dodecane than when it was administered in water.

Cell-permeable ceramides are able to block the stimulation of DNA synthesis that is induced by PA, lyso-PA [6], SPP [7] or short-chain Cer1*P* [21]. However, natural ceramides were without effect under the same conditions [6]. More recently, Ji et al. [35] showed that natural ceramides can mimic the effects of cell-permeable ceramides when they are presented to cells dissolved in a mixture of ethanol/dodecane (49:1, v/v). The kinetics of the uptake of natural ceramides by the cells [35] were consistent with those reported for fluorescent short-chain ceramide analogues,

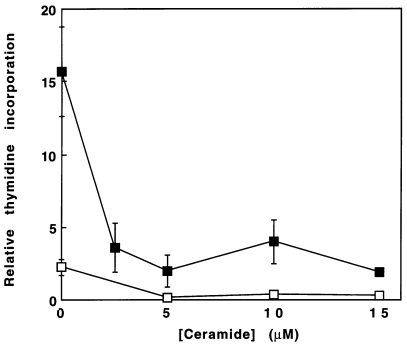

Figure 4 Inhibition of the Cer1*P*-stimulated synthesis of DNA by natural ceramides

EGFR T17 fibroblasts were incubated for 24 h in serum-free DMEM with methanol/dodecane (49:1, v/v) in the absence (□) or presence (■) of 25 μM natural Cer1*P* and the indicated concentrations of natural ceramides. [^{3}H]Thymidine was present during the last 6 h of the incubation. Results are expressed relative to the control value in the absence of methanol/dodecane and lipids, and are given as means $\pm$ range (where large enough to be shown) of two independent experiments performed in triplicate, except for the point at 10 μM ceramide where the values are means $\pm$ S.E.M. of three independent experiments. The significance of the difference between incubations with 25 μM Cer1*P* in the absence and the presence of 10 μM ceramide has a value of $P < 0.05$.

which are found to be able to insert into plasma membranes through their hydrophobic tails [36]. We now show that, under the latter conditions, natural ceramides inhibit the stimulation of DNA synthesis ($P < 0.05$; Figure 4) and block the expression of PCNA ($P < 0.02$; Figure 2 and Table 1) induced by natural Cer1*P*. Also, the cell-permeable C_2-ceramide decreases the levels of PCNA induced by C_8-Cer1*P* (Table 1). We also found that the addition of natural ceramides to fibroblasts that were prelabelled with [^{3}H]myristate increased the production of intracellular [^{3}H]ceramides. This increase was about 2.2 $\pm$ 0.4-fold in two independent experiments, which is consistent with previous work using cell-permeable ceramides [7].

Cer1*P* can be considered to be a structural analogue of PA and lyso-PA, and the mitogenic effects of the latter phospholipids have been linked to their ability to decrease cAMP levels in rat fibroblasts [37]. However, natural Cer1*P* did not alter the levels of intracellular cAMP significantly. The relative cAMP concentration was 0.96 $\pm$ 0.11 (mean $\pm$ range of two independent experiments performed in quadruplicate) compared with the control value, which is in agreement with previous work with short-chain Cer1*P* [21].

Many mitogenic agents, including lyso-PA and SPP, stimulate the generation of PA through the activation of PLD [6,7]. However, in contrast with lyso-PA, which increased PLD activity by 3.3 $\pm$ 0.45-fold (mean $\pm$ S.E.M. of seven independent experiments), the relative PLD activity in the presence of Cer1*P* was 0.81 $\pm$ 0.06 (mean $\pm$ range of two independent experiments performed in triplicate) when compared with the control value. This is also the case for short-chain Cer1*P* [21], suggesting that activation of PLD is not essential for the induction of DNA synthesis. Also, in contrast with the effects of PA, lyso-PA or EGF, neither natural Cer1*P* nor C_8-Cer1*P* was able to alter the levels of cellular tyrosine phosphorylation (results not shown) or the phosphorylation state of MAP kinase within 5–30 min (Figure 5).

Further experiments were designed to test whether natural Cer1*P* could stimulate the expression of c-*fos* or c-*myc*, which are induced upon stimulation by mitogens or cytokines in diverse

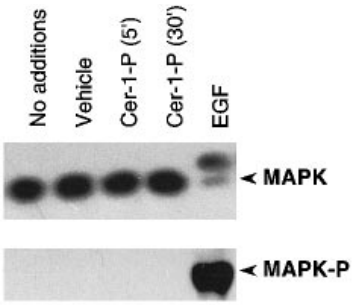

Figure 5 Lack of stimulation of MAP kinase phosphorylation by natural Cer1*P*

EGFR T17 fibroblasts were incubated for 24 h in DMEM containing 0.1% foetal calf serum, and then for 5 min without or with methanol/dodecane (49:1, v/v) alone (vehicle), natural Cer1*P* (25 μM; for 5 or 30 min) or EGF (10 nM). Lysates (30 μg of protein) of the fibroblasts were analysed by SDS/PAGE, and Western blotting was performed as described in the Materials and methods section. The upper panel shows an immunoblot using an anti-(MAP kinase) antibody, and the lower panel shows an immunoblot using an anti-(phospho-MAP kinase) antibody. Results are representative of four independent experiments.

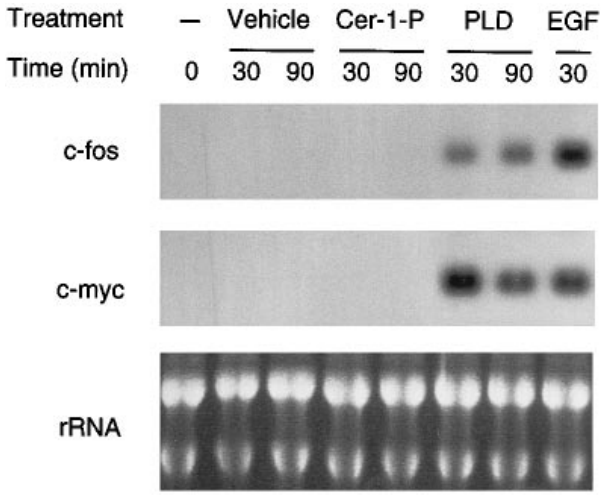

Figure 6 Cer1*P* does not affect c-*fos* or c-*myc* gene expression

EGFR T17 fibroblasts were incubated for 24 h in DMEM containing 0.1% foetal calf serum, and then without or with methanol/dodecane (49:1, v/v) alone (vehicle), natural Cer1*P* (25 μM), PLD (5 units/ml) or EGF (10 nM) for 30 or 90 min. Northern blot analysis was performed with 30 μg of total RNA. Filters were sequentially hybridized with c-*fos* and c-*myc* probes. Relative amounts of RNA loaded in each lane were visualized by staining the gel with ethidium bromide (bottom panel). Results shown are representative of three separate experiments.

cell types [38]. Figure 6 shows that, in contrast with EGF or exogenous bacterial PLD (which was used to generate PA at the plasma membrane of cells), natural Cer1*P* failed to induce the expression of c-*fos* and c-*myc* mRNAs. The effects of natural Cer1*P* were also determined between 1 and 24 h, and no significant changes in the expression of these genes were observed.

Ca^{2+} ions have also been shown to be involved in the induction of the early events of mitogenesis [39]. Therefore we tested to see whether Cer1*P* could modify the intracellular concentration of Ca^{2+}. Natural Cer1*P* could not be used to test Ca^{2+} mobilization, since the solvent mixture of methanol/dodecane (49:1, v/v) in which it was dissolved interfered with the fluorescent probe, and this made the interpretation of results difficult. Instead we used mitogenic concentrations of C_8-Cer1*P*, which was sonicated in water. In contrast with lyso-PA, C_8-Cer1*P* did not elevate the intracellular concentration of Ca^{2+} (results not shown).

DISCUSSION

The identification of ceramide kinase [9,40] and phosphatase [18–20] in cells indicates that ceramides and Cer1*P* can be interconverted. These findings, together with the observation that synthetic short-chain Cer1*P* can stimulate cell proliferation [21], raised the possibility that natural Cer1*P* might be important in controlling cell activation. However, natural Cer1*P* when added to the medium sonicated in water failed to stimulate the synthesis of DNA in fibroblasts [21]. It was reported recently [35] that natural ceramides become cell-permeant when they are dissolved in ethanol/dodecane (49:1, v/v). In the present work, we synthesized Cer1*P* from a sample of natural ceramides. However, the Cer1*P* that we produced was not soluble in this solvent mixture. This could have been due to a decrease in the hydrophobicity of Cer1*P* compared with ceramide. Therefore we replaced ethanol with methanol, a less hydrophobic alcohol. This change was sufficient to bring natural Cer1*P* into solution, and it also helped dispersion of the phospholipid into aqueous solutions. Under these conditions, natural Cer1*P* interacted readily with fibroblasts and stimulated the incorporation of [^{3}H]thymidine into DNA. Furthermore, Cer1*P* enhanced the expression of PCNA, a cell-cycle regulator that is present at sites of ongoing DNA synthesis [22]. It is possible that methanol/dodecane (49:1, v/v) facilitates the insertion of Cer1*P* into the plasma membrane of cells, thus allowing interaction of the phospholipid with elements that are required for synthesis of DNA and expression of PCNA. Radiolabelled [^{32}P]Cer1*P*, when added to the incubation medium sonicated in water, also interacted with the fibroblasts. However, there was a lack of response to natural Cer1*P*. This might be due to the inability of Cer1*P* to insert appropriately into the plasma membrane of cells in the absence of dodecane, as indicated for natural ceramide [35]. Interestingly, Cer1*P* is not deacylated by ceramidase activity to produce SPP [18], and [^{3}H]Cer1*P* synthesized from D-*erythro*-[^{3}H]sphingosine [21] does not generate significant amounts of [^{3}H]SPP or [^{3}H]sphingosine, which are also mitogenic for fibroblasts [7,41]. We have not detected in the present work any conversion of natural [^{32}P]Cer1*P* into [^{32}P]SPP; therefore the stimulation of DNA synthesis and PCNA expression that we observed were most likely elicited by the natural Cer1*P* itself.

We also demonstrate here that natural ceramides completely block the stimulation of DNA synthesis and the expression of PCNA induced by natural Cer1*P*. These are important findings, since they implicate natural ceramides and Cer1*P* in regulation of the cell cycle. Our results also suggest that the generation of ceramides from sphingomyelin, and the activities of ceramide kinase and ceramide phosphatase, may be tightly coupled to control the level of the signal that prevails in a cell under specific circumstances. Therefore, increased concentrations of ceramides relative to Cer1*P* would cause the cell to enter apoptosis, whereas relatively higher concentrations of Cer1*P* compared with ceramides would stimulate DNA synthesis and PCNA expression. The inhibitory effects of natural ceramides are unlikely to be caused solely by a physical interaction with Cer1*P*, since 2.5 μM ceramide almost completely blocked the effect of 25 μM Cer1*P* on DNA synthesis. However, exogenous ceramide can also increase the production of intracellular ceramide, which could potentiate its anti-mitogenic effects. Activation of PLD may not be essential for the induction of DNA synthesis or PCNA expression (i.e. by Cer1*P*). This observation also indicates that

the inhibition of PLD by ceramides may not be sufficient for their anti-mitogenic effects.

Cer1*P* is a structural analogue of PA, and therefore it could act through similar mechanisms to cause cell activation. However, unlike PA or lyso-PA, concentrations of natural Cer1*P* that stimulate DNA synthesis did not increase PLD activity, nor did they decrease cAMP concentrations. It is known that tyrosine phosphorylation of specific proteins plays a critical role in the transmission of signals leading to cell proliferation. In this respect, and in contrast with lyso-PA, which induces phosphorylation of the EGF receptor [42], natural Cer1*P* did not affect tyrosine phosphorylation. In addition, natural Cer1*P* failed to modify the phosphorylation state of MAP kinase (within 5–30 min), which has been shown to be involved in the stimulation of cell proliferation and the inhibition of apoptosis [38]. Furthermore, the induction of the immediate-early genes c-*fos* and c-*myc* is required for the stimulation of cell proliferation by a variety of growth factors and cytokines [38], sphingosine or SPP [43,44] and PA [45]. However, natural Cer1*P* failed to induce the expression of c-*fos* or c-*myc* up to 24 h of incubation. Therefore the mechanisms of action of natural Cer1*P* are not the same as those of sphingosine, SPP, PA or lyso-PA. Furthermore, natural Cer1*P* seems to be more potent in stimulating DNA synthesis (Figures 1 and 4) than PA or lyso-PA [6], sphingosine or SPP [7]. Another important difference between PA and Cer1*P* is that dephosphorylation of PA yields diacylglycerol, which can lead to PLD activation through stimulation of protein kinase C [46] and is a positive signal for cell growth [47]. In contrast, dephosphorylation of Cer1*P* produces ceramide, which inhibits PLD activity [6,7], blocks the proliferation of cells [4,5,21] and promotes apoptosis [12].

In conclusion, the present work shows for the first time that natural Cer1*P* is a potent stimulator of DNA synthesis and PCNA expression. These effects are independent of tyrosine phosphorylation or MAP kinase phosphorylation, and they do not involve PLD activation or a decrease in cAMP concentration. Furthermore, these mitogenic effects of natural Cer1*P* are independent of the expression of c-*myc* or c-*fos*, and may not involve changes in intracellular Ca^{2+} concentrations. The stimulation of DNA synthesis and PCNA expression induced by natural Cer1*P* can be blocked by natural ceramides, suggesting that regulation of the balance between the formation of ceramide compared with Cer1*P* may be an important factor in controlling cell activation and, in particular, cell proliferation and cell death. The use of an appropriate mixture of methanol and dodecane to deliver Cer1*P* to cells may contribute to the elucidation of the biological functions and the mechanism(s) of action of natural Cer1*P*.

We thank Dr. L. Beguinot (Hospital San Rafael, Milano, Italy) for providing the EGFR T17 cells, and Dr. L. Boscá (Institute of Biochemistry, CSIC, Madrid, Spain) for his help in determining intracellular Ca^{2+} concentrations. We are also grateful to Dr. D. N. Brindley and Dr. J. M. Mato for helpful discussions. This work was supported by grants from Dirección General de Investigación, Ciencia y Tecnología (DGICYT), Comunidad Autónoma de Madrid, and Europharma (Boehringer Ingelheim group). L. M. F. is a fellow of Europharma.

REFERENCES

1 Hannun, Y. A. (1994) J. Biol. Chem. **264**, 3125–3128
2 Kolesnick, R. and Golde, D. W. (1994) Cell **77**, 325–328
3 Heller, R. A. and Krönke, M. (1994) J. Cell Biol. **126**, 5–9
4 Okazaki, T., Bielawska, A., Bell, R. M. and Hannun, Y. A. (1990) J. Biol. Chem. **265**, 15823–15831
5 Hannun, Y. A., Obeid, L. M. and Wolff, R. A. (1993) Adv. Lipid Res. **25**, 43–65
6 Gomez-Muñoz, A., Martin, A., O'Brien, L. and Brindley, D. N. (1994) J. Biol. Chem. **269**, 8937–8943
7 Gomez-Muñoz, A., Waggoner, D. W., O'Brien, L. and Brindley, D. N. (1995) J. Biol. Chem. **270**, 26318–26325
8 Kim, M. Y., Linardic, C., Obeid, L. and Hannun, Y. A. (1991) J. Biol. Chem. **266**, 484–489
9 Mathias, S., Dressler, K. A. and Kolesnick, R. N. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 10009–10013
10 Dobrowsky, R. T. and Hannun, Y. A. (1992) J. Biol. Chem. **267**, 5048–5051
11 Goldkorn, T., Dressler, A., Muindi, J., Radin, N. S., Mendelsohn, D., Menaldino, D., Liotta, D. and Kolesnick, R. N. (1991) J. Biol. Chem. **266**, 16092–16097
12 Obeid, L. M., Linardic, C. M., Karolak, L. A. and Hannun, Y. A. (1993) Science **259**, 1769–1771
13 Hayakawa, M., Jayadev, S., Tsujimoto, M., Hannun, Y. A. and Ito, F. (1996) Biochem. Biophys. Res. Commun. **220**, 681–686
14 Raines, M. A., Kolesnick, R. N. and Golde, D. W. (1993) J. Biol. Chem. **268**, 14572–14575
15 Verheij, M., Bose, M., Lin, X. H., Yao, B., Jarvis, D. W., Grant, S., Birrer, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., et al. (1996) Nature (London) **380**, 75–79
16 Kanety, H., Hemi, R., Papa, M. Z. and Karasik, A. (1996) J. Biol. Chem. **271**, 9895–9897
17 Dressler, K. A. and Kolesnick, R. N. (1990) J. Biol. Chem. **265**, 14917–14921
18 Shinghal, R., Scheller, R. H. and Bajjalieh, S. M. (1993) J. Neurochem. **61**, 2279–2285
19 Boudker, O. and Futerman, A. H. (1993) J. Biol. Chem. **268**, 22150–22155
20 Waggoner, D. W., Gómez-Muñoz, A., Dewald, J. and Brindley, D. N. (1996) J. Biol. Chem. **271**, 16506–16509
21 Gomez-Muñoz, A., Duffey, P. A., Martin, A., O'Brien, L., Byun Bittman, R. and Brindley, D. N. (1995) Mol. Pharmacol. **47**, 883–889
22 Dietrich, D. R. (1993) Crit. Rev. Toxicol. **23**, 77–109
23 Velu, T. J., Beguinot, L., Vass, W. C., Willingham, M. C., Merlino, G. T., Pastan, I. and Lowy, D. R. (1987) Science **238**, 1408–1410
24 Schneider, E. G. and Kennedy, E. P. (1973) J. Biol. Chem. **248**, 3739–3741
25 Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E. and Bell, R. M. (1986) J. Biol. Chem **261**, 8597–8600
26 Tovey, K. C., Oldham, K. G. and Wheland, J. A. M. (1974) Clin. Chim. Acta **56**, 211–234
27 Liscovitch, M., Chalifa, V., Danin, M. and Eli, Y. (1991) Biochem. J. **279**, 319–321
28 Martin, A., Gomez-Muñoz, A., Waggoner, D. W., Stone, J. C. and Brindley, D. N. (1993) J. Biol. Chem. **268**, 23924–23932
29 Grynkiewicz, G., Poenic, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
30 Junco, M., Diaz-Guerra, M. J. and Boscá, L. (1993) Endocrinology **132**, 309–318
31 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
32 Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 5201–5205
33 Auverx, J. A., Staels, B. and Sassone-Corsi, P. (1990) Nucleic Acids Res. **18**, 221–228
34 Stanton, L. W., Watt, R. and Marcu, KB. (1983) Nature (London) **303**, 401–406
35 Ji, L., Zhang, G., Uematsu, S., Akahori, Y. and Hirabayashi, Y. (1995) FEBS Lett. **358**, 211–214
36 Pagano, R. E. and Sleight, R. G. (1985) Science **229**, 1051–1057
37 van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T. and Moolenaar, W. H. (1989) Cell **59**, 45–54
38 Hale, A. J., Smith, C. A., Sutherland, L. C., Stoneman, V. E. A., Longthorne, V. L., Culhane, A. C. and Williams, G. T. (1996) Eur. J. Biochem. **236**, 1–26
39 Zhang, H., Buckley, N. E., Gibson, K. and Spiegel, S. (1990) J. Biol. Chem. **265**, 76–81
40 Kolesnick, R. N. and Hemer, M. R. (1990) J. Biol. Chem. **265**, 18803–18808
41 Zhang, H., Desai, N. N., Olivera, S., Seki, T., Brooker, G. and Spiegel, S. (1991) J. Cell Biol. **114**, 155–167
42 Daub, H., Weiss, F. U., Wallasch, C. and Ullrich, A. (1996) Nature (London) **379**, 557–564
43 Su, Y., Rosenthal, D., Smulson, M. and Spiegel, S. (1994) J. Biol. Chem. **269**, 16512–16517
44 Berger, A., Bittman, R., Schmidt, R. R. and Spiegel, S. (1996) Mol. Pharmacol. **50**, 451–457
45 Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J. and de Laat, S. W. (1986) Nature (London) **323**, 171–173
46 Liscovitch, M. and Chalifa, V. (1994) in Signal-Activated Phospholipases (Liscovitch, M., ed.), pp. 31–63, R. G. Landes Co., Austin, TX
47 Merrill, Jr., A. H. and Stevens, V. L. (1989) Biochim. Biophys. Acta **1010**, 131–139

Received 15 October 1996/14 February 1997; accepted 11 March 1997