Extracellular sphingosine 1-phosphate stimulates formation of ethanolamine from phosphatidylethanolamine: modulation of sphingosine 1-phosphate-induced mitogenesis by ethanolamine

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In this work, we determined the effects of sphingosine 1-phosphate (S1P) on phospholipase D (PLD)-mediated hydrolysis of phosphatidylethanolamine (PtdEtn), and evaluated the effects of the water-soluble product ethanolamine on S1P-induced DNA synthesis in NIH 3T3 cells. In [¹⁴C]ethanolamine-labelled cells, S1P (0.5–5 μ M) stimulated PLD-mediated hydrolysis of PtdEtn 1.5–2.1-fold. Down-regulation of protein kinase C by chronic (24 h) treatment of cells with 300 nM PMA, or pretreatments (10 min) with the cell-permeant calcium chelator 1,2-bis-(*O*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid tetra-acetoxymethyl ester led to the inhibition of S1P-induced PtdEtn hydrolysis. S1P alone was a weak inducer of DNA synthesis, but its effects were enhanced by phosphocholine (PCho), insulin, ATP or PMA. Ethanolamine (5–100 μ M) did not modify the mitogenic effect of S1P alone, whereas at 50–100 μ M concentrations it actually enhanced the mitogenic effect of PCho via a

mitogen-activated protein (MAP) kinase-independent mechanism. In contrast, 5–20 μ M concentrations of ethanolamine, which correspond to normal blood ethanolamine levels in humans, strongly inhibited DNA synthesis induced by S1P plus PCho via a MAP kinase-dependent mechanism; importantly, less or no inhibition was observed with 50–100 μ M concentrations of ethanolamine. At 5–50 μ M concentrations, ethanolamine also inhibited the synergistic mitogenic effects of both S1P plus insulin (22–27% inhibition) and PCho plus ATP (45–73% inhibition) but not those of S1P plus PMA or S1P plus ATP. The results indicate that S1P stimulates PLD-mediated hydrolysis of PtdEtn by a mechanism that may involve a regulatory protein kinase C isoform. Increased formation of ethanolamine by PLD-mediated PtdEtn hydrolysis or by other means may be required for maximal stimulation of DNA synthesis by S1P in the presence of insulin, and particularly PCho.

INTRODUCTION

Sphingosine 1-phosphate (S1P) is a derivative of sphingosine that has been shown to play a role in growth-factor signal transduction [1–3] and in the prevention of ceramide-mediated programmed cell death [4]. Work in this [5] and other laboratories [1,6,7] has revealed that S1P alone is a relatively weak mitogen, and that its full mitogenic activity requires the presence of either growth factors such as insulin [2,5] or platelet-derived growth factor [8], or co-mitogens like phosphocholine (PCho) or ATP [5]. It also has been established that the mitogenic action of S1P involves activation of mitogen-activated protein (MAP) kinase [9].

Phospholipase D (PLD), hydrolysing phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn) or both, is present in most animal cells examined so far [10–14]. Some of the primary and secondary lipid and water-soluble products of PLD-mediated phospholipid hydrolysis have effects on cell growth when directly added to the incubation medium (reviewed in [10–14]). Furthermore, the usually very low basal activity of PLD can be enhanced by numerous growth factors [10,11,15], activators of protein kinase C (PKC), such as PMA [13], and sphingosine [16–20]. All these observations suggest that PLD may be a component of the signal-transducing apparatus involved

in cell-growth regulation. However, as yet no proof has been provided to support a role of PLD in the regulation of mitogenesis.

So far, only relatively few laboratories have reported stimulatory effects of S1P on PLD activity. In Swiss 3T3 fibroblasts [21], bovine pulmonary artery endothelial cells [20] and rat fibroblasts [22], S1P was found to stimulate either the PLD-mediated transphosphatidyl reaction or PtdCho hydrolysis. These studies have not revealed any possible relationship between the actions of S1P on PLD activity and mitogenesis.

In this work we examined the effects of S1P on PtdEtn hydrolysis as well as the possible role of the water-soluble product ethanolamine in regulating S1P-induced DNA synthesis in NIH 3T3 cells. S1P was found to stimulate the formation of ethanolamine from PtdEtn. Furthermore, physiologically relevant concentrations of ethanolamine significantly altered S1P-induced mitogenesis in the presence of PCho and to a lesser extent in the presence of insulin, PMA or ATP.

EXPERIMENTAL

Materials

PMA, PCho, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sphingosine, thapsigargin and Dowex-

Abbreviations used: S1P, sphingosine 1-phosphate; PtdCho, phosphocholine; PLD, phospholipase D; PtdEtn, phosphatidylethanolamine; PtdEtnOH, phosphatidylethanol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BAPTA-AM, 1,2-bis-(*O*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid tetra-acetoxymethyl ester; PKC, protein kinase C; MAP, mitogen-activated protein; MEK, MAP kinase kinase.

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50W (H⁺ form) were purchased from Sigma; S1P and 1,2-bis-(*O*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid tetra-acetoxymethyl ester (BAPTA-AM) were obtained from Biomol; PD 098059 and ionomycin were purchased from Calbiochem; [*methyl*-¹⁴C]choline chloride (50 mCi/mmol), [²⁻¹⁴C]ethanolamine (60 mCi/mmol), and [¹⁻¹⁴C]palmitic acid (60 mCi/mmol) were bought from Amersham; [*methyl*-³H]thymidine (500 mCi/mmol) was purchased from Dupont NEN; insulin was bought from Boehringer Mannheim; tissue-culture reagents were purchased from Gibco-BRL; and phosphatidylethanol (PtdEtOH) was from Avanti Polar Lipids.

Cell culture

NIH 3T3 clone-7 fibroblasts, obtained from Dr. Douglas R. Lowy (National Cancer Institute, Bethesda, MD, U.S.A.), were continuously cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-calf serum, penicillin/streptomycin/neomycin (50 µg/ml, 50 µg/ml and 100 µg/ml respectively) and glutamine (2 mM).

Cell-viability assay

Changes in the number of viable cells upon treatments with S1P or sphingosine were determined by the MTT method [23] with the modifications described by Carmichael et al. [24]. A Lab-system Multiskan MS microplate reader equipped with software and computer was used to read A_{540} and to analyse data.

Labelling of cellular DNA with [³H]thymidine

NIH 3T3 cells were grown in 12-well tissue-culture dishes to about 40% confluency in the presence of 10% serum, washed, and then incubated in serum-free medium for 24 h. The cells (≈ 70–80% confluent) were washed again and then treated (in serum-free medium) for 16 h with S1P in the absence or presence of PCho, insulin, PMA, ATP and/or ethanolamine; in each case S1P was added 10 min before the other agents. When the MAP kinase kinase (MEK) inhibitor PD 098059 was used, it was added to the incubation medium 60 min before S1P. After treatments, incubation of cells was continued in the presence of [*methyl*-³H]thymidine (1.0 µCi/well) for 60 min. The cells were washed twice with PBS, then four times with 5% (v/v) trichloroacetic acid, and finally twice with absolute ethanol. The acid-insoluble material was redissolved in 0.3 M NaOH, and an aliquot was taken to measure DNA-associated ³H radioactivity in a liquid-scintillation counter.

Measurement of PtdEtOH formation in NIH 3T3 cells

Cells were grown in 6-well tissue-culture dishes for 48 h to ≈ 70–80% confluency and labelled with [¹⁴C]palmitic acid (0.4 µCi/ml) for the last 24 h. On a molar basis, the ratio of ¹⁴C radioactivity in the cellular pools of substrate PtdCho and PtdEtn was ≈ 2:1. Cells were washed and then incubated in fresh medium for an additional 2 h (to lower the amount of unesterified labelled palmitic acid). Finally, the medium was replaced with Hepes-buffered physiological saline solution (nominally calcium free), followed by treatment of cells (at 37 °C) first with 10 µM BAPTA-AM for 10 min, and then with S1P and/or PMA for 15 min (in the continuous presence of BAPTA-AM, when applicable) in the presence of 150 mM ethanol. At the conclusion of incubations, cells were scraped into 2 ml of ice-cold methanol followed by rapid transfer of the methanol extract to 2 ml of chloroform. After phase separation, PtdEtOH present in the

chloroform phase was separated from other phospholipids on 1% potassium oxalate-impregnated silica gel H plates (Analtech) with a solvent system of chloroform/methanol/acetone/acetic acid/water (50:10:15:10:2, by vol.).

Determination of PLD-mediated formation of [¹⁴C]choline and [¹⁴C]ethanolamine from prelabelled phospholipids

NIH 3T3 cells, grown in 12-well plates, were labelled with [¹⁴C]choline (0.45 µCi/ml) or [¹⁴C]ethanolamine (0.35 µCi/ml) for 48 h. At the end of the labelling period, the cultures were ≈ 70–80% confluent. The cells were washed and then incubated for 3 h in fresh medium to complete incorporation of remaining radioactive precursors into cellular phospholipids. The medium was replaced with fresh medium containing only 1% fetal-calf serum and supplemented with 20 mM choline or 2 mM ethanolamine, as appropriate. (In the presence of 10% serum the 'basal' activities of PLD were 20–25% higher, and the effects of S1P were correspondingly smaller, compared with activities in 1% serum, probably reflecting the presence of some S1P in the serum.) Unlabelled choline and ethanolamine served to block further metabolism of newly formed [¹⁴C]choline and [¹⁴C]ethanolamine respectively, as described previously [25]. Cells were incubated in the presence of sphingosine, S1P and/or PMA for 15 min at 37 °C, followed by the termination of incubations as described above. In the experiments described in Table 1, incubations in Hepes-buffered physiological saline solution and treatments with BAPTA-AM were performed as described above. After phase separation, choline and ethanolamine metabolites were separated on Dowex-50(H⁺)-packed columns (Bio-Rad Econo-columns; 1 ml bed volume) with minor modifications of the procedure described by Cook and Wakelam [26]. The initial flow-through (4.5 ml) along with a following 3.5 or 5 ml water wash contained glycerophosphoethanolamine or glycerophosphocholine respectively. Phosphoethanolamine and PCho were eluted by 15 and 20 ml of water respectively. Finally, ethanolamine and choline were eluted by 12 ml and 20 ml of 1 M HCl respectively. The values at the beginning of treatments represented about 20–25% or 30–37% of [¹⁴C]choline or [¹⁴C]ethanolamine formed during a 15 min incubation period in the absence of PLD activators; these background values, which showed the above variations depending on whether a 10 min preincubation period was involved or not, were not significantly affected by BAPTA-AM. The metabolites of [¹⁴C]ethanolamine and [¹⁴C]choline were further identified by TLC, and phospholipids were separated as previously indicated [27].

Data analysis

Statistical analyses were performed with Student's *t* test. Results are expressed either as means ± S.E.M. for the stated number of separate experiments or as means ± S.D. for the stated number of determinations (separate wells) in a single experiment. Statistical significance was determined at the 0.05 level.

RESULTS

Stimulatory effects of S1P on the hydrolysis of PtdCho and PtdEtn

In [¹⁴C]ethanolamine-prelabelled NIH 3T3 cells, previously characterized activators of PLD, including sphingosine and PMA, stimulated the formation of [¹⁴C]ethanolamine by PLD-mediated hydrolysis of [¹⁴C]PtdEtn [14,16,18,27,28]. Accordingly, [¹⁴C]ethanolamine-labelled NIH 3T3 cells were used to determine the effects of S1P on PtdEtn-hydrolysing PLD activity.

Figure 1 S1P stimulates PtdEtn hydrolysis in the absence, but not in the presence, of PMA

Cells were prelabelled with [¹⁴C]ethanolamine for 48 h, and then were treated with 0.5–5 μM S1P (A) or 5–50 μM sphingosine (B) in the absence (●) or presence (▲) of 100 nM PMA for 15 min. Each point represents the mean ± S.E.M. of three separate experiments (*n* = 9). Asterisks indicate results significantly (*P* < 0.05–0.01 levels) different *from the untreated control or **from the value obtained with PMA alone.

As a reference, in the same experiment we also usually determined the effect of either sphingosine or PMA. The incubation time (15 min) and the maximal concentrations of S1P (5 μM) and sphingosine (50 μM) were selected so that these compounds were interacting with the cells without causing any detectable cytotoxic effects determined by Trypan Blue exclusion and by the MTT method. Furthermore, in preliminary experiments we determined that in the presence of 1% serum maximal effects of S1P on PtdEtn hydrolysis can be observed after 15 min incubation (results not shown).

Treatment of [¹⁴C]ethanolamine-labelled cells with S1P resulted in increased hydrolysis of [¹⁴C]PtdEtn; about 2-fold stimulatory effects required 2–5 μM concentrations of S1P (Figure 1A). Interestingly, S1P had practically no effects on PtdEtn hydrolysis in the co-presence of PMA, which is a more effective activator of this PLD activity (Figure 1A). In the presence of 10% serum, the effects of 1–5 μM concentrations of S1P were reduced on average by about 25%. Higher (5–50 μM) concentrations of sphingosine also stimulated PtdEtn hydrolysis (Figure 1B), as reported previously [16,18]. However, in sharp contrast with S1P, sphingosine and PMA synergistically stimulated PtdEtn hydrolysis (Figure 1B). Overall, data shown in Figure 1 indicated that added S1P stimulates PtdEtn hydrolysis and that its effect is different from that of sphingosine, at least in the co-presence of PMA.

When [¹⁴C]ethanolamine-labelled cells were treated with 5 μM S1P or 100 nM PMA in the absence of unlabelled ethanolamine, about 25–30% less [¹⁴C]ethanolamine, and correspondingly more [¹⁴C]phosphoethanolamine, was formed. However, both PLD activators retained their ability to enhance significantly [¹⁴C]ethanolamine formation. Strikingly, after stimulation of cells in an ethanolamine-free medium with either S1P or PMA, about 95% of newly formed [¹⁴C]ethanolamine was present in the incubation medium (results not shown). These data indicate that ethanolamine, formed upon PLD-mediated hydrolysis of PtdEtn, is

almost quantitatively released from cells when the incubation medium contains little or no ethanolamine.

Previously we have shown that down-regulation of calcium-dependent PKC isoforms by prolonged treatment of NIH 3T3 cells with PMA decreased, but did not abolish, the stimulatory effect of sphingosine on PtdEtn hydrolysis [18]. In the present work, we observed that a treatment of NIH 3T3 cells with 300 nM PMA for 24 h completely eliminated the stimulatory effect of S1P on PtdEtn hydrolysis. This suggested that the effect of S1P on PtdEtn hydrolysis could be mediated by a calcium-dependent PKC isoform; this warranted an examination of possible calcium dependence of S1P effect.

Effects of BAPTA-AM on S1P-induced PLD activity

S1P is known to mobilize calcium from intracellular stores [29–32], which could lead to partial activation of the regulatory PKC isoform and subsequent stimulation of PLD activity. If this is the case, then BAPTA-AM, a chelator that can penetrate the cell membrane and bind intracellular free calcium, should inhibit the effect of S1P on PtdEtn hydrolysis. This possibility was examined next. To estimate the length of the optimal pretreatment period and the effective concentration of BAPTA-AM, we examined the effects of this chelator on PMA-induced PtdEtn hydrolysis. Since the effects of PMA on this PLD activity are mediated by the calcium-dependent PKC-α isoform [28], the rate of inhibition of PMA-stimulated PtdEtn hydrolysis by BAPTA-AM was expected to provide an indirect measure of how effectively BAPTA-AM can reduce the intracellular level of free calcium. We observed that, after treating the cells with 10 μM BAPTA-AM (a non-cytotoxic concentration in shorter-term experiments) for 10 min in the nominally calcium-free Hepes-buffered saline medium, the subsequent stimulatory effect of PMA on PtdEtn hydrolysis (observed over a 15 min incubation

Table 1 Inhibition of S1P- and PMA-induced PtdEtn and PtdCho hydrolysis as well as PtdEtOH synthesis by BAPTA-AM in NIH 3T3 cells

Cells, prelabelled with [¹⁴C]ethanolamine or [¹⁴C]choline for 48 h, or with [¹⁴C]palmitic acid for 24 h, were treated with 10 μM BAPTA-AM, 5 μM S1P or 100 nM PMA in Hepes-buffered physiological saline medium as described in the Experimental section. Values are either the means ± S.D. of four determinations in a single experiment representative of four (columns 1–4), or are the means ± S.E.M. of three separate experiments (*n* = 8) (columns 5 and 6).

Addition	Formation of product (d.p.m./15 min per 10 ⁶ d.p.m. of [¹⁴ C]phospholipid)					
	[¹⁴ C]Ethanolamine		[¹⁴ C]Choline		[¹⁴ C]PtdEtOH	
	– BAPTA	+ BAPTA	– BAPTA	+ BAPTA	– BAPTA	+ BAPTA
None	8 160 ± 970	7 640 ± 610	3 970 ± 280	3 250 ± 210	1 150 ± 290	990 ± 180
S1P	19 460 ± 1660	12 440 ± 1980	14 880 ± 1300	9 360 ± 1140	2 640 ± 380	1 670 ± 270
PMA	23 190 ± 2040	13 560 ± 1420	22 120 ± 2330	10 970 ± 930	3 880 ± 410	2 060 ± 205

Figure 2 S1P stimulates PtdCho hydrolysis in the absence, but not in the presence, of PMA

Cells were prelabelled with [¹⁴C]choline for 48 h, and then were treated with 0.5–5 μM S1P (A) or 5–50 μM sphingosine (B) in the absence (●) or presence (▲) of 100 nM PMA for 15 min as described in the Experimental section. Each point represents the mean ± S.E.M. of three separate experiments (*n* = 9). Asterisks indicate results significantly (*P* < 0.01 level) different *from untreated control, or **from the value obtained with PMA alone.

period) was reduced by about 64 % (Table 1). Increasing the length of the pretreatment period up to 20 min did not result in greater inhibition of PMA effect. Using the same protocol we found that BAPTA-AM also inhibited the stimulatory effect of S1P on PtdEtn hydrolysis by about 62 % (Table 1).

In [¹⁴C]choline-labelled NIH 3T3 cells S1P (0.5–5 μM) also stimulated the hydrolysis of [¹⁴C]PtdCho (Figure 2A). Sphingosine and S1P caused similar increases in the activity of PtdCho-hydrolysing PLD activity, but the effects of sphingosine required about 10-fold higher concentrations. Again, the effects of S1P and PMA were clearly non-additive (Figure 2A), whereas the effects of sphingosine and PMA were additive (Figure 2B). Chronic (24 h) treatment of cells with 300 nM PMA abolished (results not shown), whereas a 10 min pretreatment with BAPTA-AM inhibited (by 50 %), the stimulatory effect of S1P on PtdCho hydrolysis (Table 1).

Formation of phosphatidylalcohol from substrate phospholipids and an alcohol, like ethanol, by the transphosphatidylation

reaction is a specific function of PLD. In [¹⁴C]palmitic acid-labelled fibroblasts a pretreatment with BAPTA-AM did not modify the basal level of [¹⁴C]PtdEtOH formation from prelabelled phospholipids and ethanol. However, this chelator significantly decreased the stimulatory effects of both S1P and PMA on the synthesis of [¹⁴C]PtdEtOH (Table 1). We also examined the effects of ionomycin and thapsigargin, agents that are capable of increasing the level of cytoplasmic calcium, on PtdEtn and PtdCho hydrolysis. Ionomycin used at 1–5 μM concentrations increased PtdEtn and PtdCho hydrolysis by 1.5–1.7-fold. However, thapsigargin (0.1–2 μM) had no detectable effects on either PtdEtn or PtdCho hydrolysis (results not shown).

Modulation of S1P-induced DNA synthesis by ethanolamine

Stimulation of phospholipid hydrolysis by S1P could represent a significant event as far as it relates to the biological effects of S1P

Figure 3 Combined effects of S1P, ethanolamine and PCho on DNA synthesis

Serum-starved (24 h) NIH 3T3 cells were first treated for 10 min with 0–5 μ M concentrations of S1P, followed by incubations for 16 h (in the continuous presence of S1P) in the absence (●) or presence of 20 μ M ethanolamine (▲) or 1 mM PCho (■). Each point represents the mean $\pm$ S.D. of three determinations in a single experiment representative of two.

such as regulation of mitogenesis. Although evidence was previously reported to suggest that certain primary and secondary products of PLD action, including phosphatidic acid, lyso-phosphatidic acid and PCho, could be involved in the regulation of mitogenesis (reviewed in [14]), practically nothing was known about the possible regulatory role of physiological concentrations of ethanolamine. This led us to examine whether ethanolamine has any effects on S1P-induced mitogenesis in the presence of agents, including PCho, insulin and ATP, which were previously shown to enhance the effects of S1P on DNA synthesis [5].

The concentration of ethanolamine in the circulation is between 5 and 50 μ M [33,34], which may increase via stimulated formation of ethanolamine from PtdEtn. We assumed that if ethanolamine is used at 5–100 μ M concentrations, this will cover the physiological concentration range of this intermediate. As shown in Figure 3, S1P alone had a moderate stimulatory effect on DNA synthesis, with a $\approx$ 9-fold (maximal) stimulatory effect requiring 5 μ M concentration. Ethanolamine, added at 20 μ M (Figure 3) or 100 μ M concentration (not shown), did not modify the effect of S1P alone. However, in agreement with our previous findings [5], 1 mM PCho greatly enhanced the mitogenic effect of S1P (Figure 3). In addition, in the presence of PCho 2.5 μ M S1P was maximally effective in stimulating DNA synthesis (Figure 3).

Possible modification of the combined mitogenic effects of S1P and PCho by 5–100 μ M concentrations of ethanolamine was examined at 0.25, 1 and 2.5 μ M concentrations of S1P. At each concentration of S1P, 5–20 μ M ethanolamine clearly inhibited the synergistic stimulatory effects of S1P and PCho on DNA synthesis (Figure 4A). In the presence of 0.25 μ M S1P, the inhibitory effects of 5–20 μ M ethanolamine were relatively larger (47–60 % inhibition) than in the presence of 2.5 μ M S1P (30–33 %

Figure 4 Biphasic effects of ethanolamine on DNA synthesis induced by S1P in combination with PCho and zinc

Serum-starved (24 h) NIH 3T3 cells were first incubated for 3 h in the absence (A) or presence (B) of 25 μ M ZnCl_2 , followed by treatments for 16 h with 0–100 μ M concentrations of ethanolamine in the presence of 0.25 μ M S1P plus 1 mM PCho (●), 1 μ M S1P plus 1 mM PCho (▲), or 2.5 μ M S1P plus 1 mM PCho (■). S1P was added 10 min before the other agents, whereas PCho and ethanolamine were added to the cells simultaneously. Values are the means $\pm$ S.E.M. of three separate experiments ($n = 9$).

Figure 5 Synergistic stimulation of DNA synthesis by PCho and ethanolamine

Serum-starved NIH 3T3 cells were treated for 16 h with ethanolamine (0–250 μ M) in the absence (●) or presence (▲) of 1 mM PCho. Each point represents the mean $\pm$ S.D. of triplicate determinations in a single experiment representative of three.

inhibition). Ethanolamine had less inhibitory effects at 50 μ M concentration, and in each case 100 μ M ethanolamine slightly enhanced the combined effects of S1P and PCho (Figure 4A). The inhibitory action of ethanolamine was observed only when it was added before (1–5 min) or simultaneously with PCho. When ethanolamine was added 20 s after PCho, little or no inhibitory effects of ethanolamine were observed.

Zinc, an important regulator of cell growth [35], is present in the periphery in the 10–20 μ M concentration range [35–37], which can be doubled under certain conditions, including stress [37]. Thus it was considered important to test whether the synergistic effects of S1P and PCho as well as the inhibitory effects of ethanolamine still can be observed in the presence of zinc. As shown in Figure 4(B), zinc (25 μ M) actually enhanced the synergistic stimulatory effects of S1P and PCho, and it also slightly promoted the inhibitory effects of 5–20 μ M ethanolamine. It is important to point out that, in the presence of zinc and PCho, 2.5 μ M S1P stimulated DNA synthesis to about the same level that can be achieved by 10 % serum (which induces maximal DNA synthesis in these cells).

Ethanolamine, particularly in the 50–250 μ M concentration range, enhanced the small mitogenic effect of 1 mM PCho in the absence of S1P (Figure 5). This finding, coupled with the previous observation that ethanolamine did not affect the effect of S1P alone (Figure 3), suggested that ethanolamine somehow interacted with the PCho component. To verify this possibility, next we examined the effects of ethanolamine on the previously characterized [5] synergistic mitogenic effects of PCho and ATP in the absence of S1P. As shown in Figure 6, ethanolamine inhibited the combined effects of PCho and ATP, although with some different characteristics. In particular, whereas in the presence of S1P plus PCho about 5 μ M ethanolamine was sufficient to inhibit maximally DNA synthesis, in the presence of PCho and ATP maximal inhibition required 50 μ M ethanolamine. How-

Figure 6 Inhibitory effects of ethanolamine on PCho-plus-ATP-induced DNA synthesis

Serum-starved NIH 3T3 fibroblasts were treated for 16 h with ethanolamine (0–250 μ M) plus 100 μ M ATP plus 1 mM PCho in the absence (●) or presence (▲) of 25 μ M zinc. Treatments were started with zinc for 3 h, followed by ATP for 10 min, and then continued in the presence of PCho and ethanolamine for 16 h. Values are the means $\pm$ S.E.M. of three separate experiments ($n = 9$).

Table 2 Effects of PD 098059 on DNA synthesis induced by combinations of S1P, PCho and ethanolamine

Serum-starved NIH 3T3 cells were first treated with 50 μ M PD 098059 for 60 min followed by treatments with 5 μ M S1P, 1 mM PCho and/or 100 μ M ethanolamine as indicated. S1P was added 10 min before PCho and ethanolamine. Values are the means $\pm$ S.D. of triplicate determinations in a single experiment representative of three.

Addition	Incorporation of [3 H]thymidine into DNA (d.p.m./60 min per 10^6 cells)	
	– PD 098059	+ PD 098059
PCho	3490 $\pm$ 280	3240 $\pm$ 300
S1P	14810 $\pm$ 2030	3740 $\pm$ 410
S1P + PCho	207880 $\pm$ 16940	39710 $\pm$ 5200
PCho + ethanolamine	12660 $\pm$ 1470	13980 $\pm$ 1730
S1P + PCho + ethanolamine	239920 $\pm$ 13530	53770 $\pm$ 4880

ever, in both cases the concentration-dependent effects of ethanolamine were biphasic. In addition, zinc clearly increased the inhibitory potential of ethanolamine on PCho-plus-ATP-induced DNA synthesis (Figure 6).

Since S1P was reported to stimulate MAP kinase activity [9], we were interested to know to what extent S1P-plus-PCho- as well as PCho-plus-ethanolamine-stimulated DNA synthesis involves this enzyme activity. The recently described specific inhibitor of MEK, PD 098059 [38], was used for this purpose, based on the finding that activation of MAP kinase requires its phosphorylation by MEK (reviewed in [38]). As shown in Table 2, PD 098059 strongly inhibited the effect of S1P alone as well as the combined effects of S1P and PCho on DNA synthesis regardless of the presence of 100 μ M ethanolamine. In contrast,

Figure 7 Inhibitory effects of ethanolamine on S1P-plus-insulin-induced DNA synthesis

Serum-starved NIH 3T3 cells were treated for 16 h with ethanolamine (0–100 μM) in the presence of 500 nM insulin (●) or insulin plus 2.5 μM S1P (▲). S1P was added 10 min before insulin and ethanolamine. Values are the means ± S.E.M. of three separate experiments ($n = 9$).

Figure 9 Modulatory effects of ethanolamine on PMA-plus-S1P-induced DNA synthesis

Serum-starved NIH 3T3 cells were treated for 16 h with ethanolamine (0–100 μM) in the presence of 100 nM PMA (●), PMA plus 0.25 μM S1P (▲), or PMA plus 2 μM S1P (■). S1P was added 10 min before PMA and 20 min before ethanolamine. Values are the means ± S.E.M. of three separate experiments ($n = 9$).

Figure 8 Modulatory effects of ethanolamine on S1P-plus-ATP-induced DNA synthesis

Serum-starved NIH 3T3 cells were treated for 16 h with ethanolamine (0–100 μM) in the presence of 100 μM ATP plus 0.25 μM S1P (●), ATP plus 0.5 μM S1P (▲), or ATP plus 2.5 μM S1P (■). S1P was added 10 min before ATP or ethanolamine. Values are the means ± S.E.M. of three separate experiments ($n = 9$).

PD 098059 had practically no effect on PCho-plus-ethanolamine-induced DNA synthesis.

Previously, S1P and insulin were also shown to stimulate synergistically DNA synthesis [5]. As indicated in Figure 7, at 5 and 20 μM concentrations ethanolamine inhibited S1P- (2.5 μM) plus-insulin-induced DNA synthesis by 22–27 %, whereas 50 and

100 μM ethanolamine had no inhibitory effects. Thus lower concentrations of ethanolamine are clearly more effective inhibitors of S1P-induced DNA synthesis in the presence of PCho than in the presence of insulin.

In contrast with the large potentiating effect of ATP on PCho-induced DNA synthesis (Figure 6), the effects of 0.25–2.5 μM S1P were only doubled in the presence of ATP (Figure 8). Furthermore, the combined stimulatory effects of S1P and ATP were slightly enhanced, rather than inhibited, by 20–100 μM concentrations of ethanolamine. PMA (100 nM) and S1P also synergistically stimulated DNA synthesis (Figure 9). Again, ethanolamine (20–100 μM) slightly enhanced the effects of PMA in both the absence and presence of S1P (Figure 9).

DISCUSSION

Stimulatory effects of S1P on PtdCho-hydrolysing PLD activity have previously been demonstrated in several cell lines [20–22]. Here, we have shown that in NIH 3T3 fibroblasts S1P can stimulate PLD-mediated hydrolysis of both PtdEtn and PtdCho. Chronic treatment of cells with PMA, which results in the down-regulation of PKC-α [39], abolished the stimulatory effects of S1P on PtdEtn (and PtdCho) hydrolysis. This implicates PKC-α, a major regulator of PtdEtn hydrolysis [28], as a possible mediator of S1P action. In turn, PKC-α may be partially activated by calcium. On the one hand, this possibility is supported by findings reported from several laboratories that S1P increases the level of cytoplasmic calcium [29–32], and by our demonstration that the cell-permeant calcium chelator BAPTA-AM reduces the effects of S1P on PtdEtn (and PtdCho) hydrolysis. On the other hand, it should be emphasized that ionomycin only partially mimicked the effect of S1P, whereas thapsigargin had no effect at all. Clearly, more experiments are required to rule out possible non-specific effects of BAPTA-AM and to prove that calcium indeed plays a role in the mediation of S1P effects on PtdEtn hydrolysis.

Although many mitogens stimulate PLD activity, research in this area has generally failed to provide information with regard to the role of PLD activation in mitogenesis. However, recent evidence suggests that PCho, a secondary product of PLD-mediated PtdCho hydrolysis, may serve as a co-mitogen when mitogens or other co-mitogens are present. As was originally reported by Cuadrado et al. [40], PCho may be required for the actions of several growth factors. Work in our laboratory provided further evidence that PCho, in combination with insulin, ATP or S1P, can have powerful effects on DNA synthesis [5]. Thus increased formation of choline through S1P-induced hydrolysis of PtdCho, associated with the phosphorylation of choline and the release of PCho by an ATP-regulated process [5], could lead to the potentiation of mitogenic effects of S1P. Of course, such a mechanism would require that S1P acts through cell-surface receptors. Indeed, several laboratories have recently demonstrated the existence of cell-surface S1P receptors [8,41–43].

Another water-soluble product of PLD action that may play a role in cell-growth regulation is ethanolamine. Previously, our laboratory reported that in NIH 3T3 fibroblasts ethanolamine and its methylated analogues can enhance the mitogenic effect of insulin and insulin-like growth factor I [44–46]. These co-mitogenic effects required relatively high, 0.25–1 mM, concentrations of ethanolamines. Presently, we observed biphasic effects of ethanolamine on S1P-plus-PCho-induced DNA synthesis in the 5–100 μ M concentration range of ethanolamine. These latter effects of ethanolamine are likely to be more physiological, because in normal human subjects the blood ethanolamine concentration is in the range of 5–20 μ M, and it increases only 2–3-fold in certain pathological conditions [33]. Also, in rats the mean concentration of ethanolamine in the serum was shown to be about 28 μ M, which was increased, although only up to the 50–55 μ M level, during a 10–22 h period following partial hepatectomy [34]. Incidentally, in our experiments the same concentrations of ethanolamine that were found in the blood of healthy humans exhibited inhibitory effects on S1P-plus-PCho-induced mitogenesis in NIH 3T3 cells. Conversely, elevation of ethanolamine concentration to 100 μ M restored the mitogenic activity of S1P in the presence of PCho. Clearly, if certain human cells are also regulated by these agents, then at normal blood ethanolamine levels the combined mitogenic effect of S1P and PCho would be expected to be well below the possible maximal value. However, activators of PLD, perhaps including S1P alone or in combination with other activators, could significantly enhance the formation of ethanolamine, resulting in higher ethanolamine concentrations in the extracellular space. This may turn out to be a potentially important new mechanism to control mitogenesis that merits further investigation.

Physiologically relevant concentrations of ethanolamine also inhibited, although only moderately, the large synergistic mitogenic effects of S1P and insulin, whereas the combined effects of S1P and ATP as well as S1P and PMA were slightly enhanced, rather than inhibited, by ethanolamine. On the other hand, the previously characterized large synergistic mitogenic effects of PCho and ATP [5] were also significantly inhibited by 5–50 μ M concentrations of ethanolamine. These data suggest that physiological concentrations of ethanolamine may exert modulatory, mostly inhibitory, effects on the regulation of mitogenesis. Furthermore, activation of PtdEtn-specific PLD, presumably resulting in higher extracellular concentrations of ethanolamine, may result in partial or full reversal of the inhibitory ethanolamine effects.

At present, the biochemical basis accounting for the biphasic effects of ethanolamine on S1P-plus-PCho-induced DNA syn-

thesis is not entirely clear. The experiments with the MEK inhibitor PD 098059 indicate that PCho and S1P synergistically activate MAP kinase activity regardless of the presence of 100 μ M ethanolamine. Thus the loss of the inhibitory potency of ethanolamine at higher concentrations does not occur because of a shift from a MAP kinase-dependent to a MAP kinase-independent mechanism. Instead, ethanolamine may primarily affect coupling of the PCho-generated signal to the potentiating signals generated by S1P or ATP. This would be consistent with findings, obtained so far, that major alteration of DNA synthesis by ethanolamine can only be observed in the co-presence of PCho. More studies are required to determine the mechanism(s) of these potentially important effects of ethanolamine on mitogenesis.

At least three major issues will have to be resolved before the role of ethanolamine in the regulation of mitogenesis can be determined. First, it will be necessary to determine the site of action(s) of ethanolamine. Since the action of S1P on mitogenesis seems to occur via an extracellular site [5] probably involving cell-surface S1P receptors [8,41–43], it is likely that ethanolamine also acts extracellularly. In this context it is important to note that ethanolamine, formed in S1P- or PMA-treated cells, is rapidly and quantitatively released from the cells to the medium. Clearly, if ethanolamine acts through an extracellular site, then regulation of ethanolamine levels in the blood and in the extracellular space in various tissues becomes an important issue. The limited amount of information available in the literature [33,34] indicates that certain physiological conditions indeed can up-regulate the blood ethanolamine level. Secondly, it will be important to identify those human cell types that respond to S1P and ethanolamine in a manner similar to that observed in NIH 3T3 fibroblasts. Related studies in this laboratory have so far revealed that in Swiss 3T3 fibroblasts and JB6 epidermal cells S1P and PCho also synergistically stimulate DNA synthesis, and that low concentrations of ethanolamine inhibit their effects. This suggests that the observed interactions among the mitogenic effects of S1P, PCho and ethanolamine may be widespread. Thirdly, it will be necessary to determine the signal-transduction mechanism(s) involved in the mitogenic actions of S1P and the biphasic modulatory effects of ethanolamine. As discussed above, our results indicate that S1P and PCho in combination can greatly stimulate MAP kinase activity, but the biphasic effects of ethanolamine may involve other mechanisms.

In summary, we have shown that treatment of NIH 3T3 fibroblasts with S1P results in the stimulation of PLD-mediated hydrolysis of PtdEtn and PtdCho. In addition, our results suggest the existence of novel mechanisms by which changes in ethanolamine levels in the blood and/or in the extracellular space in various tissues might modulate the mitogenic activity of S1P in the presence of other growth regulatory agents. It remains to be seen whether specific human cells also respond to S1P and ethanolamine in a manner described for NIH 3T3 cells. It also remains to be determined whether S1P and/or other activators of PLD, or certain hitherto unrecognized physiological conditions, can shift the concentration of ethanolamine in the blood and/or in the extracellular space in tissues to a level that permits full activation of DNA synthesis by S1P.

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