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QUARTERLY

# Exogenous sphingosine 1-phosphate and sphingosylphosphorylcholine do not stimulate phospholipase D in C6 glioma cells*°

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**In the present study we investigate the effect of exogenous sphingosine, sphingosine 1-phosphate and sphingosylphosphorylcholine on phospholipase D (PLD) activity in glioma C6 cells. The cells were prelabeled with [1-$^{14}$C]palmitic acid and PLD-mediated synthesis of [$^{14}$C]phosphatidylethanol was measured. Sphingosine 1-phosphate and sphingosylphosphorylcholine did not stimulate [$^{14}$C]phosphatidylethanol formation either at low (0.1–10 $\mu$M) or high (25–100 $\mu$M) concentrations. On the other hand, sphingosine at concentrations of 100–250 $\mu$M strongly stimulated PLD activity as compared to the effect of phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), known as a PLD activator. The effect of TPA on PLD is linked to the activation of protein kinase C. The present study also shows that sphingosine additively enhances TPA-mediated PLD activity. This is in contrast to the postulated role of sphingosine as a protein kinase C inhibitor. These results demonstrate that in glioma C6 cells sphingosine not only affects PLD independently of its effect on protein kinase C, but also is unable to block TPA-mediated PLD activity.**

Phospholipase D (PLD) is the enzyme hydrolyzing predominantly phosphatidylcholine in a reaction generating choline and phosphatidic acid [1]. Phosphatidic acid can be further hydrolyzed by phospholipase $A_2$ to form lysophosphatidic acid, a second messenger, or by

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**Abbreviations:** PEt, phosphatidylethanol; PLD, phospholipase D; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

phosphatidate phosphohydrolase to form 1,2-diacylglycerol [2]. It has been suggested that hydrolysis of phosphatidylcholine by PLD and subsequent action of phosphatidate phosphohydrolase on phosphatidic acid are responsible for the long-term increase of diacylglycerol in cells and activation of protein kinase C (PKC) [3]. Therefore, PLD has been implicated as part of a second messenger-generating system, operating in parallel with signal transduction coupled to phospholipase C and phospholipase $A_2$ [3].

The activity of PLD can be stimulated by various receptor agonists [4–7], TPA known as an activator of PKC [7, 8], and sphingosine proposed to be a physiological inhibitor of PKC [9–13]. Various products of sphingomyelin hydrolysis influence PLD activity in multiple ways. It has been found that sphingosine and the product of its phosphorylation, sphingosine 1-phosphate, enhance PLD activity [13–16], whereas sphingosylphosphorylcholine is ineffective [10] and ceramide inhibits this activity [15, 17].

Sphingosine 1-phosphate is a normal constituent of human plasma and serum where its concentration can reach 480 nM [18]. This sphingolipid acts intracellularly as a second messenger stimulating cell growth and preventing apoptosis, and as a first messenger through its recently discovered endothelial plasma membrane surface receptor Edg-1 [19]. Cloned Edg-1, involved in endothelial differentiation, is the sphingosine 1-phosphate high affinity receptor ($K_d$ = 8 nM), whereas low affinity receptors were found in platelets and F10 melanoma ($K_d$ > 100 nM) [20]. It has been suggested that the low sphingosine 1-phosphate affinity receptor can also bind lysophosphatidic acid [21, 22], and sphingosylphosphorylcholine [23].

The aim of the present study was to examine whether in glioma C6 cells sphingosylphosphorylcholine, sphingosine and sphingosine 1-phosphate can affect PLD activity in a similar manner. PLD activity was measured by phosphatidylethanol (PEt) formation. PEt is a unique phospholipid that is formed in animal cells only in the presence of ethanol *via* transphosphatidylation catalyzed exclusively by PLD [24]. We found that at low (0.1–10 $\mu$M) concentrations neither sphingosylphosphorylcholine, sphingosine nor sphingosine 1-phosphate stimulated PEt formation and hence PLD activity. However, at higher concentrations, particularly at 250 $\mu$M, sphingosine was a very strong stimulator of PLD as compared to the effect of TPA. In contrast to sphingosine, exogenous sphingosylphosphorylcholine and sphingosine 1-phosphate had no effect on PLD. We have also found that sphingosine stimulates PLD activity independently of TPA-mediated PLD activation.

## MATERIALS AND METHODS

***Materials***: C6 glioma cells were from the American Type Culture Collection (U.S.A.). Minimum Essential Medium (MEM), calf serum, trypsin solution and phosphate-buffered saline (PBS, pH 7.4) were from Gibco BRL (U.K.). Penicillin and streptomycin were from Polfa (Tarchomin, Poland). Bovine serum albumin (BSA), phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), D-(+)-erythro-*trans*-sphingosine and sphingosylphosphorylcholine and phospholipase D (from peanut) were purchased from Sigma (St. Louis, MO, U.S.A.). Sphingosine 1-phosphate was from Calbiochem (La Jolla CA, U.S.A.). [1-$^{14}$C]palmitic acid, (spec. act., 56 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). G-60 thin-layer chromatography plates were purchased from Merck (Darmstad, Germany). Phosphatidylethanol standard was prepared in our laboratory by incubation of phosphatidylcholine (from egg) with ethanol and phospholipase D from peanut (EC. 3.1.4.4.), according to Eibl & Kovatchev [25].

***Cell culture.*** Glioma C6 cells (passages 40–60) were used. The cultivation of the cells was carried out in 10 mm diameter dishes

containing 10 ml of MEM supplemented with 10% (v/v) newborn calf serum, penicillin (50 UI/ml) and streptomycin (50 $\mu$g/ml) (culture medium) under a humidified atmosphere of 5% $CO_2$ at 37°C, as previously described [26]. The cells were passed when confluent using trypsin (0.25%), and the medium was changed twice a week. The cells had reached confluence at the time of experiment.

***Assay for phospholipase D activity.*** PLD activity was assayed by measuring the formation of PEt in the presence of ethanol. C6 glioma cells were labeled by incubation with [1-$^{14}$C]palmitic acid (56 mCi/mmol, 1 $\mu$Ci/dish) for 3 h at 37°C. Calf serum was omitted from the labeling culture medium. Unincorporated [1-$^{14}$C]palmitic acid was removed by washing with ice-cold PBS. The total radioactivity of the [1-$^{14}$C]palmitic acid labeled lipids amounted, in different experiments, from 1000000 to 1500000 d.p.m./8 × $10^6$ cells. $^{14}$C-labeled cells were first preincubated for 5 min in 5 ml of the medium (culture medium without calf serum) with 150 mM ethanol, followed by a further incubation with 150 mM ethanol and with or without TPA, sphingosine, sphingosylphosphorylcholine and sphingosine 1-phosphate. Sphingosine and sphingosylphosphorylcholine were added as an equimolar complex with BSA. Sphingosine 1-phosphate, after methanol evaporation, was prepared in PBS by sonication. All additions were prepared before use. In control samples appropriate vehicles were added. All incubations were carried out at 37°C for 1 h. The reaction was terminated by the addition of 1 ml of methanol per dish. Lipids were extracted by a method of Bligh & Dyer [27], modified to contain 0.1 M HCl in the methanol. The cells were harvested by scraping into tubes to a total volume of 2 ml of methanol. Lipids were extracted by adding 2 ml of chloroform. After thorough mixing, samples were left on ice for 15 min. Phase separation was achieved by addition of 1.25 ml of 0.1 M HCl. After stirring and low-speed centrifugation, two 0.8 ml portions of the lower phase were transferred into separate Eppendorf vials, 50 $\mu$l portions were taken into scintillation vials and, after evaporation, counted for the radioactivity of total lipids, taken later as 100%. Standard of PEt was added into vials. After chloroform evaporation lipids were separated on TLC plates of Silica Gel 60 using solvent system consisting of hexane/diethyl ether/acetic acid (60:40:1.6, by vol.). PEt was identified by comparing its $R_F$ values with those of the standard. The lipids were visualized by exposure to iodine vapor. After complete evaporation of iodine from the plates the radiolabeled lipids were scraped into scintillation vials and quantitated using a Beckmann liquid scintillation counter.

***Data presentation.*** The results are mean values ±S.D. of at least triplicate determinations.

## RESULTS

### Effect of sphingosine and sphingosylphosphorylcholine on PLD activity in C6 glioma cells

Glioma C6 cells, prelabeled with [1-$^{14}$C]palmitic acid, were used to determine the effect of different concentration of sphingosine and its derivative, sphingosylphosphorylcholine, on PLD activity as compared to the known effect of TPA (Fig. 1). Sphingosine at concentrations up to 50 $\mu$M was practically without effect on [$^{14}$C]phosphatidylethanol formation, but at higher concentrations, particularly at 250 $\mu$M, it stimulated markedly the incorporation of ethanol to $^{14}$C-labeled phospholipids. On the other hand, a significant activation of PLD occurred at very low, nanomolar, concentration of TPA (Fig. 1B.). TPA was used in these experiments as a reference. As it is shown, 250 $\mu$M sphingosine was even more effective in PEt formation than TPA (Fig. 1). On the contrary, sphingosylphosphorylcholine did not influence PLD activity at any concentration examined (Fig. 1A). It is

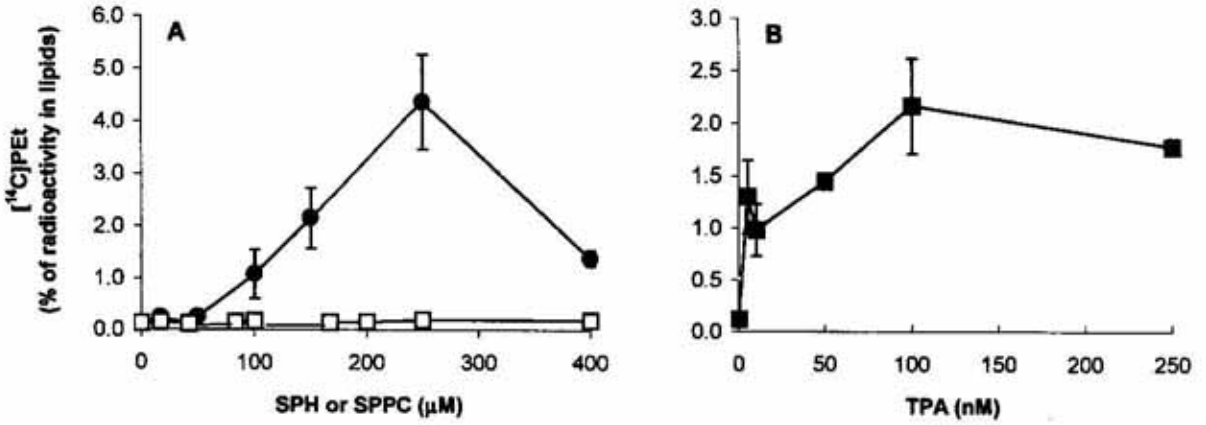


**Figure 1. Sphingosine (SPH), sphingosylphosphorylcholine (SPPC) (A) and TPA (B) concentration dependent [$^{14}$C]phosphatidylethanol formation in C6 glioma cells.**

The cells were prelabeled for 3 h with [1-$^{14}$C]palmitic acid, then preincubated for 5 min in the medium containing 150 mM ethanol, followed by addition of SPH (●), SPPC (□) or TPA (■). To the control samples appropriate vehicle was added. After 1 h incubation at 37°C lipids were extracted and [$^{14}$C]phosphatidylethanol was determined as described in Materials and Methods. Mean values ±S.D. of three experiments are shown.

worth adding that the amounts of sphingosine able to activate PLD (100–250 $\mu$M) in the present study, recalculated in nmoles per number of cells, are similar to those used in other laboratories and amounted to 6.25–15.62 nmoles sphingosine per $10^5$ cells [9–13]. Such concentrations were not cytotoxic for glioma C6 cells [28].

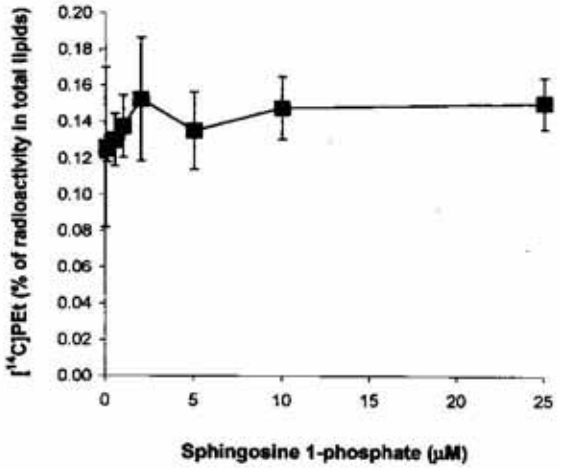


**Figure 2. Sphingosine-1-phosphate (SPP) concentration dependent [$^{14}$C]phosphatidylethanol formation in glioma C6 cells.**

Experiments were performed as described in the legend to Fig. 1. Mean values ±S.D. of two experiments are shown.

### Effect of sphinghosine 1-phosphate on PLD activity in C6 glioma cells

Sphingosine 1-phosphate, used in the concentration range between 0.1 and 10 $\mu$M, did not influence PLD activity during 1 h incubation with 150 mM ethanol (Fig. 2). There was no increase in PEt formation during 15 min incubation of C6 glioma cells with 1 $\mu$M sphingosine 1-phosphate, either (not shown). A small increase in PEt formation observed after 1 h incubation with 0.1–10 $\mu$M sphingosine 1-phosphate was statistically insignificant (Fig. 2). Moreover, the increase in sphingosine 1-phosphate concentration up to 25 $\mu$M (Fig. 2) and 100 $\mu$M (not shown) also had no effect on PEt formation. Thus, C6 glioma cells do not seem to contain sphingosine 1-phosphate-stimulated receptors able to generate PLD activation.

### The effect of simultaneous action of sphingosine and TPA on PLD activity in glioma C6 cells

It has been previously reported that sphingosine and TPA may synergistically stimulate the hydrolysis of phosphatidylcholine and

phosphatidylethanolamine in different types of cells involving both PKC-dependent and PKC-independent mechanisms [1, 7]. We have attempted to study how sphingosine affects TPA-stimulated PLD activity in glioma C6 cells. Activation of PKC by TPA is usually given as an explanation of the stimulatory effect of TPA on PLD [7].

Addition of 100 nM TPA increased PEt formation approximately 20-fold in glioma C6 cells (from 0.1 to 2.0% of [$^{14}$C]phosphatidylethanol). When the cells were incubated for 1 h with a fixed concentration of TPA (100 nM) and increasing amounts of sphingosine, the stimulatory effect of TPA and sphingosine on PLD activity was additive (Fig. 3). Similar results were obtained for 10 and 20 min of incubation (not shown). These data demonstrate that the effect of sphingosine on PLD activity in glioma C6 cells is PKC-independent.

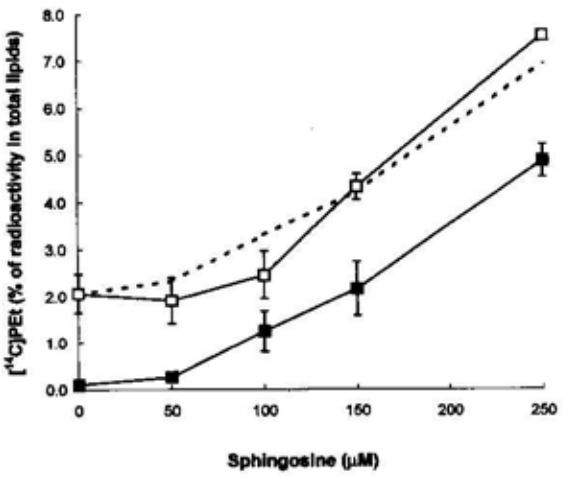


**Figure 3. Effect of the addition of increasing amounts of sphingosine (SPH) in the absence (■) and in the presence (□) of a fixed quantity (100 nM) of TPA upon the formation of [$^{14}$C]phosphatidylethanol.**

Dashed line shows the calculated sum of [$^{14}$C]phosphatidylethanol obtained separately for different concentrations of SPH and 100 nM TPA. Mean values ±S.D. of three experiments are shown.

## DISCUSSION

The present study shows that, in C6 glioma cells, extracellular sphingosine 1-phosphate and sphingosylphosphorylcholine do not stimulate formation of PEt produced by PLD in the presence of ethanol. Sphingosine 1-phosphate did not activate PLD in glioma C6 cells either at low (1–10 $\mu$M) or at high (25–100 $\mu$M) concentrations. Sphingosylphosphorylcholine was without effect on PLD activity either, at all concentrations examined. It has been reported that exogenous sphingosylphosphorylcholine does not activate PLD in glioma × neuroblastoma hybrid (NG108-15) cells [10], and sphingosine 1-phosphate, in bovine aortic endothelial cells [29] and in primary cultures of guinea-pig airway smooth muscle [12]. In contrast to these findings, PLD was activated by low concentrations of sphingosine 1-phosphate in bovine pulmonary artery endothelial cells [13], Swiss 3T3 fibroblasts [14], Rat1 and Rat2 fibroblasts [15] and NIH 3T3 fibroblasts [16].

It has been recently reported that extracellular sphingosine 1-phosphate and sphingosylphosphorylcholine activate $G_i$ protein-coupled receptor(s) in plasma membrane of various cell lines [19, 20, 23, 30]. This activation leads to increased cytoplasmic $Ca^{2+}$ concentration, inhibition of adenylyl cyclase, and opening of G protein-regulated potassium channels [23]. Sphingosine 1-phosphate has also induced $Ca^{2+}$ signals in C6 glioma cells [23, 31]; this could suggest that it might act as an agonist with its own receptor. However, PLD activity seems to be unrelated to the $G_i$ protein-coupled receptor activated by sphingosine 1-phosphate [20]. On the other hand, the possibility that the enzyme might interact with other sphingosine 1-phosphate receptors, such as sphingosine 1-phosphate/lysophosphatidic acid and sphingosine/sphingosylphosphorylcholine can not be excluded [20, 31]. Nevertheless, our data show that sphingosine 1-phosphate and sphingosylphosphorylcholine are unable in C6 glioma cells to stimulate PLD activity.

In contrast to sphingosine 1-phosphate and sphingosylphosphorylcholine, sphingosine strongly activated PLD in C6 glioma cells. However, the effect of sphingosine on PLD activity was visible only at higher concentrations. This may suggest that sphingosine does not act through plasma membrane receptor(s) but, rather, affects PLD activity intracellularly.

The mode of sphingosine action on PLD is presently unknown. The activation of PLD by sphingosine does not involve PKC since sphingosine is a PKC inhibitor. This is supported by the fact that sphingosylphosphorylcholine, which also inhibits PKC [32], had no effect on PLD activity ([10] and this study). Nevertheless, although it is generally accepted that sphingosine is a potent competitive inhibitor of PKC *in vitro*, no studies have proved sphingosine to affect PLD activity as a physiological regulator of PKC *in vivo* [33, 34]. In addition to reports demonstrating that sphingosine inhibits cellular responses dependent on PKC, a growing number of cell responses are now known to depend very little, if at all, on inhibition of PKC. A good example is the mitogenic effect of sphingosine (*via* PLD production of phosphatidic acid) on Swiss fibroblasts, which is independent of PKC [14, 35]. Our present results also show that sphingosine acts additively with TPA-dependent PLD activity in glioma C6 cells. A similar effect was observed in NIH 3T3 fibroblasts and airway smooth muscle [11, 12]. Sphingosine also enhances TPA-mediated formation of PEt in lymphocytes [36]. These results demonstrate that in some cell lines sphingosine not only affects PLD independently of PKC, but is also unable to block the TPA-mediated PLD activity.

It has been recently reported that PKC$\alpha$ and PKC$\beta$ can directly (*via* their regulatory domains) activate PLD in an ATP-independent manner but other PKC isoenzymes are ineffective [1, 7]. Thus, one can speculate that, in glioma C6 cells, sphingosine does not participate in this process and therefore does not inhibit the TPA-dependent PLD activity.

In conclusion, the present study shows that, in glioma C6 cells, sphingosine 1-phosphate and sphingosylphosphorylcholine do not activate PLD, as sphingosine and TPA do. The enhancement of TPA-stimulated PLD activity by sphingosine indicates that TPA and sphingosine activate the enzyme by two different, independent, pathways and can not be involved in the same regulatory system, i.e. the PKC system. Further studies are required to elucidate and explain the molecular mechanism of these processes.

## REFERENCES

1. Exton, J.H. (1997) New developments in phospholipase D. *J. Biol. Chem.* **272**, 15579–15582.

2. Brindley, D.N. & Waggoner, D.W. (1996) Phosphatidate phosphohydrolase and signal transduction. *Chem. Phys. Lipids* **80**, 45–57.

3. Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* **9**, 484–496.

4. Billah, M.M. & Anthes, J.C. (1990) The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.* **269**, 281–291.

5. Exton, J.H. (1994) Phosphatidylcholine breakdown and signal transduction. *Biochim. Biophys. Acta* **1212**, 26–42.

6. Exton, J.H. (1990) Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* **265**, 1–4.

7. Cockroft, S. (1997) Phospholipase D: Regulation by GTPases and protein kinase C and physiological relevance. *Prog. Lipid Res.* **35**, 345–370.

8. Min, D.S. & Exton, J.H. (1998) Phospholipase D is associated in a phorbol ester-dependent

manner with protein kinase C-$\alpha$ and with a 220-kDa protein which is phosphorylated on serine and threonine. *Biochem. Biophys. Res. Commun.* **248**, 533–537.

**9.** Kiss, Z. & Anderson, W.B. (1990) ATP stimulates the hydrolysis of phosphatidylethanolamine in NIH 3T3 cells. Potentiating effect of guanosine triphosphates and sphingosine. *J. Biol. Chem.* **265**, 7345–7350.

**10.** Lavie, Y. & Liscovitch, M. (1990) Activation of phospholipase D by sphingoid bases in NG108-15 neural-derived cells. *J. Biol. Chem.* **265**, 3868–3872.

**11.** Kiss, Z. & Deli, E. (1992) Regulation of phospholipase D by sphingosine involves both protein kinase C-dependent and -independent mechanisms in NIH 3T3 fibroblasts. *Biochem. J.* **288**, 853–858.

**12.** Pyne, S., Chapman, J., Steele, L. & Pyne, N.J. (1996) Sphingomyelin-derived lipids differentially regulate the extracellular signal-regulated kinase 2 (ERK-2) and c-Jun N-terminal kinase (JNK) signal cascades in airway smooth muscle. *Eur. J. Biochem.* **237**, 819–826.

**13.** Natarajan, V., Jayaram, H.N.I., Scribner, W.M. & Garcia, J.G. (1994) Activation of endothelial cell phospholipase D by sphingosine and sphingosine-1-phosphate. *Am. J. Respir. Cell. Mol. Biol.* **11**, 221–229.

**14.** Desai, N.N., Zhang, H., Olivera, A., Mattie, M.E. & Spiegel, S. (1992) Sphingosine-1-phosphate, a metabolite of sphingosine increases phosphatidic acid levels by phospholipase D activation. *J. Biol. Chem.* **267**, 23122–23128.

**15.** Gomez-Munoz, A., Waggoner, D.W., O'Brien, L. & Brindley, D.N. (1995) Interaction of ceramides, sphingosine and sphingosine 1-phosphate in regulating DNA synthesis and phospholipase D activity. *J. Biol. Chem.* **270**, 26318–26325.

**16.** Kiss, Z., Crilly, K.S. & Anderson, W.B. (1997) Extracellular sphingosine 1-phosphate stimulates formation of ethanolamine from phosphatidylethanolamine: Modulation of sphingosine 1-phosphate-induced mitogenesis by ethanolamine. *Biochem. J.* **328**, 383–391.

**17.** Abousalham, A., Liossis, C., O'Brien, L. & Brindley, D.N. (1997) Cell-permeable ceramides prevent activation of phospholipase D by ADP-ribosylation factor and RhoA. *J. Biol. Chem.* **272**, 1069–1075.

**18.** Igarashi, Y. & Yatomi, Y. (1998) Sphingosine 1-phosphate is a blood constituent released from activated platelets, possibly playing a variety of physiological and pathological roles. *Acta Biochim. Polon.* **45**, 299–309.

**19.** Lee, M.-J., Van Brocklyn, J.R., Thangada, S., Liu, C.H., Hand, A.R., Menzeleev, R., Spiegel, S. & Hla, T. (1998) Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor Edg-1. *Science* **279**, 1552–1555.

**20.** 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.

**21.** Yatomi, Y., Yamamura, S., Ruan, F. & Igarashi, Y. (1997) Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *J. Biol. Chem.* **272**, 5291–5297.

**22.** An, S., Bleu, T., Hallmark, O.G. & Goetzl, J.E. (1998) Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J. Biol. Chem.* **273**, 7906–7910.

**23.** Van Koppen, C.J., Meyer zu Heringdorf, D., Laser, K.T., Zhang, C., Jakobs, K.H., Bunemann, M. & Pott, L. (1996) Activation of a high affinity $G_i$ protein-coupled plasma membrane receptor by sphingosine-1-phosphate. *J. Biol. Chem.* **271**, 2082–2087.

**24.** Morris, A.J., Frohman, M.A. & Engebrecht, J. (1997) Measurement of phospholipase D activity. *Anal. Biochem.* **252**, 1–9.

**25.** Eibl, H. & Kovatchev, S. (1981) Preparation of phospholipids and their analogs by phospholipase D. *Methods Enzymol.* **72**, 632–639.

**26.** Czarny, M., Sabała, P., Ucieklak, A., Kaczmarek, L. & Barańska, J. (1992). Inhibition of phosphatidylserine synthesis by glutamate, acetylcholine, thapsigargin and ionophore A23187 in glioma C6 cells. *Biochem. Biophys. Res. Commun.* **194**, 571–583.

**27.** Bligh, E.G. & Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.

**28.** Wiktorek-Wójcik, M., Banasiak, M., Czarny, M., Stępkowski, D. & Barańska, J. (1997) Serine base exchange enzyme activity is modulated by sphingosine and other amphiphilic compounds: Possible role of positive charge in increasing the synthesis of phosphatidylserine. *Biochem. Biophys. Res. Commun.* **241**, 73–78.

**29.** Meyer zu Heringdorf, D., van Koppen, C.J., Windorfer, B., Himmel, H.M. & Jakobs, K.H. (1996) Calcium signalling by G protein-coupled sphingolipid receptors in bovine aortic endothelial cells. *Naunyn Schmiedebergs Arch. Pharmacol.* **354**, 397–403.

**30.** Meyer zu Heringdorf, D., van Koppen, C.J. & Jakobs, K.H. (1997) Molecular diversity of sphingolipid signalling. *FEBS Lett.* **410**, 34–38.

**31.** Tas, P.W.L. & Koschel, K. (1998) Sphingosine-1-phosphate induces a $Ca^{2+}$ signal in primary rat astrocytes and a $Ca^{2+}$ signal and shape changes in C6 rat glioma cells. *J. Neurosci. Res.* **52**, 427–434.

**32.** Liscovitch, M. & Lavie, Y. (1991) Sphingoid bases as endogenous cationic amphiphilic "drugs". *Biochem. Pharmacol.* **42**, 2071–2075.

**33.** Hannun, Y.A., Merrill, A.H. & Bell, R.M. (1991) Use of sphingosine as inhibitor protein kinase C. *Methods Enzymol.* **201**, 316–328.

**34.** Spiegel, S. & Merrill, A. H., Jr. (1996) Sphingolipid metabolism and cell growth regulation. *FASEB J.* **10**, 1388–1397.

**35.** Zhang, H., Buckley, N.E., Gibson, K. & Spiegel, S. (1990) Sphingosine stimulates cellular proliferation *via* a protein kinase C-independent pathway. *J. Biol. Chem.* **265**, 76–81.

**36.** Cao, Y.-Z., Mastro, A.M., Eskew, M.L., Hildenbrandt, G., Reddy, P.V. & Reddy, C.C. (1995) The mechanism of sphingosine enhancement of phorbol ester-mediated phospholipase D activation in lymphocytes. *Biochem. Biophys. Res. Commun.* **217**, 908–915.