Regulation of phospholipase D by sphingosine involves both protein kinase C-dependent and -independent mechanisms in NIH 3T3 fibroblasts

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Previously, the protein kinase C (PKC) inhibitor sphingosine was found to stimulate phospholipase D (PLD)-mediated hydrolysis of both phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) in NIH 3T3 fibroblasts [Kiss & Anderson (1990) *J. Biol. Chem.* **265**, 7345–7350]. Here we examined the possible relationship between the opposite effects of sphingosine on PKC-mediated protein phosphorylation and PLD activation. After treatments for 3–5 min, sphingosine (25 μ M) and the PKC activators phorbol 12-myristate 13-acetate (PMA) (100 nM), bryostatin (100 nM) or platelet-derived growth factor (50 ng/ml) synergistically stimulated the hydrolysis of both PtdEtn and PtdCho in NIH 3T3 fibroblasts prelabelled with [14 C]ethanolamine or [14 C]choline. Inhibition of PMA-induced phospholipid hydrolysis could also be elicited by sphingosine, but this process required prolonged (60 min) treatments of fibroblasts with 40–60 μ M-sphingosine. Similarly to sphingosine, the protein phosphatase inhibitor okadaic acid also had either potentiating or inhibitory effects on PMA-stimulated PLD activity, depending on the length of incubation time and the concentration of PMA. Consistent with the presence of an inhibitory component in the overall action of PKC, the PKC inhibitor staurosporine and down-regulation of PKC activity by prolonged (24 h) treatment with PMA similarly enhanced PLD activity. Data suggest that (a) sphingosine may enhance PMA-mediated phospholipid hydrolysis by neutralizing the action of an inhibitory PKC isoform, and that (b) the stimulatory PKC isoform is less sensitive to the inhibitory action of sphingosine.

INTRODUCTION

Sphingosine has become the subject of intense investigation, primarily because of its ability to inhibit protein kinase C (PKC) [1–3]. Because PKC is a family of a variety of different isoforms with presumably different functions [4], it is often not readily apparent whether a biochemical effect of sphingosine involved inhibition of PKC or not. Stimulation of phospholipase D (PLD)-mediated hydrolysis of phosphatidylethanolamine (PtdEtn) [5] and phosphatidylcholine (PtdCho) [5,6] in NIH 3T3 fibroblasts by sphingosine is a prime example of such uncertainty. Since these processes are also stimulated by the PKC activator phorbol 12-myristate 13-acetate (PMA), it would appear that stimulation of PLD activity by sphingosine and PMA cannot involve the same regulatory system, i.e. the PKC system.

However, sphingosine could enhance PLD activity by a PKC-dependent mechanism if the overall stimulatory action of activated PKC on PLD activity involved an inhibitory component (in addition to a dominant stimulatory component) that is particularly sensitive to the inhibitory action of sphingosine. A variety of approaches, including down-regulation of PKC by prolonged treatment with PMA [7,8] and treatment of fibroblasts with the protein phosphatase inhibitor okadaic acid [9,10] or the PKC inhibitor staurosporine [11], were taken in the present study to identify this hypothetical inhibitory component of the effect of PMA on PLD activity. Results suggest that the overall stimulatory effect of PMA on PLD activity indeed consists of stimulatory and inhibitory mechanisms, and that the inhibitory mechanism is more effectively inhibited by sphingosine than is the stimulatory mechanism.

EXPERIMENTAL

Materials

PMA, sphingosine, sphingomyelinase (from *Staphylococcus aureus*) and Dowex-50W (H^+ form) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; platelet-derived growth factor (PDGF-BB; human recombinant) was from Boehringer Mannheim, Indianapolis, IN, U.S.A.; *N*-acetylsphingosine (C_{18}/C_2 ceramide) was from Matreya, Pleasant Gap, PA, U.S.A., and was also provided by Y. A. Hannun (Duke University, NC, U.S.A.); [$2-^{14}C$]ethanolamine (60 mCi/mmol), [$methyl-^{14}C$]choline chloride (50 mCi/mmol) and L -[$U-^{14}C$]serine (150 mCi/mmol) were from Amersham, Arlington Heights, IL, U.S.A. Bryostatin I was purified from the marine animal *Bugula neritina* [12], and was provided by G. R. Pettit (Cancer Research Institute, Arizona State University, Tempe, AZ, U.S.A.).

Cell culture

NIH 3T3 cells were cultured continuously in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) fetal-calf serum (GIBCO), penicillin (50 units/ml), streptomycin (50 μ g/ml) and glutamine (2 mM). Fibroblasts were seeded [$(1-2) \times 10^5$ /dish] in 100 mm-diameter plastic dishes, and growing (70–90% confluent) cell populations were used for these experiments.

Determination of water-soluble products of phospholipid degradation in NIH 3T3 cells

Cells were incubated with either [$2-^{14}C$]ethanolamine (0.25 μ Ci/ml) or [$methyl-^{14}C$]choline (0.35 μ Ci/ml) for 48 h,

Abbreviations used: PKC, protein kinase C; PLD, phospholipase D; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PMA, phorbol 12-myristate 13-acetate; PDGF, platelet-derived growth factor; C_{18}/C_2 ceramide, *N*-acetylsphingosine.

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washed, incubated in fresh medium for 3 h (to decrease the level of water-soluble ^{14}C -labelled phospholipid precursors [13]), then harvested by scraping from 4–8 dishes. Because scraping transiently enhances cellular 1,2-diacylglycerol levels in NIH 3T3 fibroblasts (discussed in [14]), we routinely inserted an additional 20 min incubation period between scraping and centrifugation (10 min at 500 g). A 30 min period is required for the return of cellular 1,2-diacylglycerol to the control level [14]. Resuspended cells were again washed, and then 0.25 ml portions $[(7-8) \times 10^5 \text{ cells/ml}]$ were incubated (final vol. 0.25 ml) in the presence of 2 mM unlabelled ethanolamine or choline, as indicated, along with other agents as specified. Incubations were terminated by addition of 4 ml of chloroform/methanol (1:1, v/v). Phase separation was initiated by addition of 3 ml of water. Fractionation of choline and ethanolamine metabolites was performed on Dowex-50W (H^+)-packed columns (Bio-Rad Econo-columns; 1 ml bed volume) as described by Cook & Wakelam [15] for the choline derivatives, with minor modifications.

The initial flow-through (4.5 ml) along with a following 3.5 ml or 5 ml water wash contained glycerophosphoethanolamine or glycerophosphocholine respectively. Ethanolamine phosphate and choline phosphate were eluted by 15 ml and 20 ml of water respectively. Finally, ethanolamine and choline were eluted by 12 ml and 20 ml of 1 M-HCl respectively. Appropriate ^{14}C -labelled standards were prepared as described previously [16]. For determination of the elution profiles of standards, 1 ml fractions were collected. The metabolites of ^{14}C ethanolamine and ^{14}C choline were further identified by t.l.c. [17]. Phospholipids were separated as described previously [18].

Determination of sphingomyelinase-mediated sphingomyelin hydrolysis

NIH 3T3 cells were incubated with $[\text{U-}^{14}\text{C}]$ serine for 48 h, washed, and incubated in fresh medium for 3 h to decrease the amount of unincorporated free ^{14}C radioactivity. Suspended cells were incubated with increasing amounts of *Staphylococcus* sphingomyelinase for 20 min. Ceramide, sphingosine and sphingomyelin were separated from other labelled phospholipids (phosphatidylserine and PtdEtn) on silica gel G plates in the solvent system chloroform/methanol/2 M- NH_3 (40:10:1, by vol.) as described by Okazaki *et al.* [19]. Sphingomyelin was further identified by two-dimensional t.l.c. as described previously [18].

RESULTS

Combined effects of sphingosine and PMA on phospholipid hydrolysis

Treatment of ^{14}C ethanolamine- or ^{14}C choline-labelled NIH 3T3 fibroblasts with PMA (100 nM) or sphingosine (25 μM) for 3 min resulted in increased degradation of both ^{14}C PtdEtn and ^{14}C PtdCho (Table 1). We have determined previously [5,16] that these concentrations of sphingosine and PMA are optimal for the stimulation of PLD-mediated hydrolysis of phospholipids. Importantly, at this early time point sphingosine and PMA had synergistic stimulatory effects on the hydrolysis of both PtdEtn and, to a lesser extent, PtdCho (Table 1).

The experiment shown in Table 1 was also repeated with fibroblasts which were double-labelled with ^{14}C ethanolamine and ^{14}C choline for 48 h. In this case, 1 ml fractions of 1 M-HCl eluate were collected, with about 90 % of ^{14}C ethanolamine and 80 % of ^{14}C choline being eluted between fractions 3–7 and 8–13 respectively. In double-labelled cells, treatments with sphingosine plus PMA for 3 min resulted in synergistic effects on the formation of both ^{14}C -labelled products similar to that described

in Table 1 (results not shown). In addition, using t.l.c. for the verification of ^{14}C -labelled products [17], we found that in sphingosine and PMA-treated ^{14}C ethanolamine-labelled cells the ^{14}C ethanolamine fraction (12 ml) contained less than 10 % choline. This is not surprising, because in NIH 3T3 fibroblasts methylation of PtdEtn is a relatively minor reaction; in ^{14}C ethanolamine-labelled fibroblasts PtdEtn contains about 20 times more ^{14}C label than does PtdCho (Z. Kiss & E. Deli, unpublished work).

At longer incubation times (7.5–30 min), the synergistic effects of sphingosine and PMA showed either a decreasing tendency (PtdEtn hydrolysis; Fig. 1a) or rapidly disappeared (PtdCho hydrolysis; Fig. 1b). When the incubation time was extended up to 60 min, PMA-stimulated hydrolysis of both PtdEtn (Fig. 2a) and PtdCho (Fig. 2b) was inhibited by 40 and 60 μM concentrations of sphingosine, with some inhibition of PtdCho hydrolysis by 20 μM -sphingosine being also observable.

Enhancement of bryostatin- and PDGF-stimulated phospholipid hydrolysis by sphingosine

Bryostatin is another activator of PKC [20–23], which also stimulates PLD-mediated phospholipid hydrolysis [17]. As shown in Table 2, bryostatin and sphingosine had clear synergistic stimulatory effects on the hydrolysis of both PtdEtn and PtdCho when incubations were conducted for 10 min or less. When the incubation period was extended up to 20–30 min, these agents had only additive (20 min) or less than additive effects (30 min) (results not shown).

Recently the mitogenic peptide PDGF was shown to stimulate PtdEtn hydrolysis in NIH 3T3 fibroblasts [24]. In the present work, the stimulatory effects of PDGF and sphingosine on PtdEtn hydrolysis were clearly more than additive at a short (3 min) incubation time (Table 3). Again, after longer (10–20 min) incubation periods, the stimulatory effects of PDGF and sphingosine were only additive (10 min) or less than additive (20 min) (results not shown).

If the delayed inhibition of PMA-induced hydrolysis of phospholipids by high concentrations of sphingosine involved inhibition of PKC activity, it is difficult to explain the rapid stimulatory effects of lower concentrations of sphingosine. Since in the human epidermoid A431 cell the stimulatory effect of sphingosine on phosphorylation of the epidermal-growth-factor receptor is apparently mediated by ceramide [25], an agent which does not inhibit PKC activity, we next examined whether ceramide can also mediate the effect of sphingosine on phospholipid hydrolysis in fibroblasts.

Comparison of the stimulatory effects of sphingosine, sphingomyelinase and C_{18}/C_2 ceramide on the hydrolysis of PtdEtn

To determine the possible role of ceramide in mediation of the effect of sphingosine on PtdEtn hydrolysis, the cellular level of ceramide was raised by either sphingomyelinase or the cell-permeant ceramide analogue C_{18}/C_2 ceramide. As shown in Fig. 3, addition of 250–400 m-units of sphingomyelinase/ml to NIH 3T3 fibroblasts (labelled with ^{14}C serine until radioisotopic equilibrium was achieved) for 20 min resulted in hydrolysis of 35–40 % of cellular sphingomyelin. Increased ^{14}C sphingomyelin hydrolysis was accompanied by a proportional increase in the cellular level of labelled ceramide, with no significant formation of labelled sphingosine being detectable (Fig. 3).

Despite the ability of sphingomyelinase (250–400 m-units/ml) to raise the cellular content of ceramide 3–3.3-fold, these concentrations of sphingomyelinase enhanced the formation of ^{14}C ethanolamine from the prelabelled PtdEtn pool only about 1.7-fold at most (Fig. 4). Similarly, 5–30 μM concentrations of

Table 1. Rapid synergistic stimulatory effects of sphingosine and PMA on the hydrolysis of PtdEtn and PtdCho in NIH 3T3 fibroblasts

Fibroblasts were labelled with [14 C]ethanolamine or [14 C]choline for 48 h as described in the Experimental section, followed by treatments for 3 min with sphingosine (25 μ M) and/or PMA (100 nM). The mean 14 C content of PtdEtn and PtdCho was 1.56×10^6 and 1.13×10^6 d.p.m./ 10^6 cells respectively. Data are means $\pm$ S.E.M. of four incubations. This experiment was repeated twice with similar results.

Addition	Formation of 14 C-labelled products (d.p.m./3 min per 10^6 d.p.m. of [14 C]phospholipid)	
	[14 C]Ethanolamine	[14 C]Choline
None	295 $\pm$ 80	440 $\pm$ 75
Sphingosine	2650 $\pm$ 440	1850 $\pm$ 625
PMA	1810 $\pm$ 410	2360 $\pm$ 380
Sphingosine + PMA	7030 $\pm$ 510	5610 $\pm$ 570

Fig. 1. Combined stimulatory effects of sphingosine and PMA on the hydrolysis of PtdEtn and PtdCho in NIH 3T3 fibroblasts

Fibroblasts were prelabelled with [14 C]ethanolamine (a) or [14 C]choline (b) for 48 h, followed by incubation of suspended cells for 7.5–30 min in the absence (●) or presence of 20 μ M-sphingosine (■), 100 nM-PMA (▲), or sphingosine plus PMA (◆). The mean 14 C content of PtdEtn and PtdCho was 1.68×10^6 and 1.22×10^6 d.p.m./ 10^6 cells respectively. Each point represents the mean $\pm$ S.E.M. of four incubations. Similar results were obtained in six other experiments.

Table 2. Synergistic stimulatory effects of sphingosine and bryostatin on the hydrolysis of PtdEtn and PtdCho in NIH 3T3 fibroblasts

Fibroblasts, prelabelled with [14 C]ethanolamine or [14 C]choline as described in the Experimental section, were treated with sphingosine (25 μ M) and/or bryostatin (100 nM) for 5 or 10 min. Data are means $\pm$ S.E.M. of four incubations. Similar results were obtained in another experiment.

Addition	Formation of 14 C-labelled products (d.p.m./ 10^6 d.p.m. of [14 C]phospholipid)			
	[14 C]Ethanolamine		[14 C]Choline	
	5 min	10 min	5 min	10 min
None	410 $\pm$ 110	880 $\pm$ 130	460 $\pm$ 45	980 $\pm$ 115
Sphingosine	2910 $\pm$ 260	4230 $\pm$ 420	2210 $\pm$ 365	3140 $\pm$ 510
Bryostatin	1350 $\pm$ 130	2850 $\pm$ 175	1290 $\pm$ 170	3060 $\pm$ 550
Sphingosine + bryostatin	6630 $\pm$ 230	8385 $\pm$ 305	5515 $\pm$ 415	8750 $\pm$ 650

Fig. 2. Inhibition of PMA-induced phospholipid hydrolysis by higher concentrations of sphingosine

Fibroblasts were prelabelled with [14 C]ethanolamine (a) or [14 C]choline (b) for 48 h, followed by incubation of suspended cells for 60 min with 0–60 μ M concentrations of sphingosine in the absence (●) or presence (▲) of 100 nM-PMA. Each point represents the mean $\pm$ S.E.M. of four incubations. Similar results were obtained in two other experiments.

Table 3. Stimulation of PtdEtn hydrolysis by sphingosine and PDGF

Fibroblasts were labelled with [14 C]ethanolamine for 48 h as described in the Experimental section, followed by treatments for 3 min with sphingosine (25 μ M) and/or PDGF (50 ng/ml). Data are means $\pm$ S.E.M. of four incubations. This experiment was repeated once with similar results.

	Formation of [14 C]ethanolamine (d.p.m./3 min per 10^6 d.p.m. of [14 C]PtdEtn)	
	No sphingosine	+ Sphingosine
None	450 $\pm$ 95	2995 $\pm$ 400
+ PDGF	1020 $\pm$ 180	4985 $\pm$ 385

C_{18}/C_2 ceramide had only very small (> 1.4 -fold) stimulatory effects on PtdEtn hydrolysis (Fig. 4). In addition, the small stimulatory effects of both sphingomyelinase and C_{18}/C_2 ceramide were less than additive with that of PMA after 5 min incubation (results not shown). Finally, neither sphingomyelinase (100–400 m-units/ml) nor C_{18}/C_2 ceramide (10–30 μ M) had any significant stimulatory effects on PtdCho hydrolysis (results not shown). On the basis of these results, we conclude that ceramide does not mediate the stimulatory effect of sphingosine on phospholipid hydrolysis.

Effect of okadaic acid on PMA-induced phospholipid hydrolysis

For the interpretation of the apparently complex effects of sphingosine on PLD activity, it was important to determine the possible role of protein phosphorylation in the regulation of this enzyme activity. For this, okadaic acid, a powerful inhibitor of major serine/threonine protein phosphatases [9,10], was used under several conditions. When treatments of prelabelled cells with 100 nM-PMA were performed for only 30 min, 2 μ M-okadaic acid had no reproducible effects on PMA-induced hydrolysis of PtdEtn or PtdCho (results not shown). After 1 h treatment, okadaic acid slightly (but not statistically significantly) inhibited PtdEtn hydrolysis induced by 100 nM-PMA (Fig. 5a), and it also

Fig. 3. Effect of sphingomyelinase on the degradation of sphingomyelin in NIH 3T3 fibroblasts

Fibroblasts were prelabelled with [14 C]serine for 48 h, followed by incubation of suspended cells for 20 min in the presence of various concentrations of *Staphylococcus* sphingomyelinase, as described in the Experimental section. Sphingomyelin (■), ceramide (▲) and sphingosine (●) were separated by t.l.c. Each time point represents the mean $\pm$ S.E.M. of four incubations. Similar results were obtained in another experiment.

Fig. 4. Concentration-dependent effects of sphingosine, C_{18}/C_2 ceramide and sphingomyelinase on the hydrolysis of PtdCho in NIH 3T3 fibroblasts

Fibroblasts were prelabelled with [14 C]ethanolamine for 48 h, and then suspended cells were incubated for 15 min in the presence of various concentrations of C_{18}/C_2 ceramide (●), sphingomyelinase (■) or sphingosine (▲), as described in the Experimental section. [14 C]ethanolamine was separated by ion-exchange chromatography. The 14 C content of PtdEtn was 1.55×10^6 d.p.m./ 10^6 cells. Each point represents the mean $\pm$ S.E.M. of four incubations. Similar results were obtained in two other experiments.

slightly enhanced PtdCho hydrolysis (Fig. 5b). After treatment for 2 h, however, okadaic acid clearly inhibited the effect of PMA on the hydrolysis of both PtdEtn (Fig. 5a) and, to a lesser extent, PtdCho (Fig. 5b). These results suggest that the major effect of okadaic acid is to decrease, rather than increase, PLD activity.

One reason for our inability to demonstrate strong positive

Fig. 5. Effects of okadaic acid on the hydrolysis of phospholipids in NIH 3T3 fibroblasts

Fibroblasts, prelabelled with [14 C]ethanolamine (a) or [14 C]choline (b) for 48 h, were incubated for 1 or 2 h in the absence (□) or presence of $2 \mu\text{M}$ -okadaic acid (▨), 100 nM -PMA (▨) or okadaic acid plus PMA (■) as indicated. Data represent means $\pm$ S.E.M. of four incubations. Similar results were obtained in two other experiments.

Fig. 6. Modulation of PMA-induced PtdEtn hydrolysis by okadaic acid

[14 C]ethanolamine-prelabelled fibroblasts were treated with 0–40 nM concentrations of PMA in the absence (●) or presence (▲) of $2 \mu\text{M}$ -okadaic acid for 1 h as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. of four incubations. Similarly small stimulatory effects were obtained with okadaic acid in two other experiments.

regulation of PLD activity by okadaic acid (i.e. by protein phosphorylation) could be that, in the presence of 100 nM -PMA, PKC was fully activated (in contrast with the inhibitory isoform), resulting in full phosphorylation of the regulatory protein in question. Thus we next examined the effect of okadaic acid on PtdEtn hydrolysis at sub-optimal concentrations of PMA. As shown in Fig. 6, okadaic acid enhanced only slightly, although consistently (in three experiments), the stimulatory effects of 10 and 20 nM concentrations of PMA on PtdEtn hydrolysis. These data suggested again that protein phosphorylation may also positively regulate PLD activity. However, it was clear that

Fig. 7. Comparison of the effects of staurosporine, PMA and sphingosine on PtdEtn hydrolysis in control and PMA-pretreated NIH 3T3 fibroblasts

Fibroblasts were labelled with [¹⁴C]ethanolamine for 48 h in the absence (a) or presence (b) of 300 nM-PMA during the last 24 h of the labelling period, as described in the Experimental section. Prelabelled fibroblasts were further incubated for 20 min in the absence (□) or presence of 1 μM-staurosporine (▨), 100 nM-PMA (▩) or staurosporine plus PMA (■). If present, the concentration of sphingosine was 25 μM. Data are means ± S.E.M. of four incubations. Similar results were obtained in three other experiments.

experiments with okadaic acid could provide only limited information, because of the possible co-existence of the stimulatory and inhibitory effects of protein phosphorylation, and because of the fact that some protein phosphatases are not inhibited by 2 μM-okadaic acid [9]. To have more information on the possible role of protein phosphorylation in the regulation of PLD activity by sphingosine, we next compared the effects of sphingosine with that of staurosporine, another PKC inhibitor [11], in untreated fibroblasts and in fibroblasts in which PKC activity was down-regulated.

Comparison of the effects of staurosporine, PMA and sphingosine on PLD activity in untreated and PMA-pretreated fibroblasts

Addition of staurosporine to [¹⁴C]ethanolamine-labelled fibroblasts resulted in about 2-fold stimulation of PtdEtn hydrolysis (Fig. 7a). Importantly, staurosporine failed to enhance further the effect of sphingosine, suggesting that stimulation of PLD activity by these agents involved a common mechanism. In addition to its stimulatory effect, staurosporine also decreased the stimulatory effects of PMA and PMA plus sphingosine (Fig. 7a).

Prolonged (24 h) pretreatment of fibroblasts with 300 nM-PMA, an established procedure to down-regulate PKC activity in fibroblasts [7,8], also enhanced PtdEtn hydrolysis about 2-fold (Fig. 7b). In PMA-pretreated fibroblasts, staurosporine or PMA had no additional stimulatory effects (Fig. 7b). In contrast, whereas enhanced PLD activity in PMA-pretreated cells was accompanied by a corresponding decrease in the fold stimulatory effect of sphingosine, this agent still stimulated PtdEtn hydrolysis about 2-fold (instead of 4-fold) in these cells (Fig. 7b).

DISCUSSION

Results obtained by using a variety of pharmacological tools suggest that sphingosine may act on PLD-mediated phospholipid hydrolysis through three different mechanisms, as depicted

below, where (S)PKC and (I)PKC are stimulatory and inhibitory PKC isoforms respectively:

sphingosine inhibits (S)PKC → PLD ↓ (1)

sphingosine → PLD ↑ (2)

sphingosine inhibits (I)PKC → PLD ↑ (3)

Treatment of cells with high (40–60 μM) concentrations of sphingosine for a sufficiently long period of time results in the inhibition of PMA-induced hydrolysis of both PtdCho and PtdEtn (mechanism 1). Although it appears that sphingosine may act on the hydrolysis of these phospholipids in a somewhat different time- and concentration-dependent manner which merits further investigation, this consideration is beyond the scope of this investigation. What we want to stress here is the inability of sphingosine to inhibit PMA-induced PLD activity at earlier incubation times, which raises the possibility that stimulation of PLD activity by PKC activators may not involve a protein-phosphorylation reaction. Previous observations from our and other laboratories support this possibility. Thus we observed [17] that addition of PMA to membranes from HL60 cells maximally stimulated PLD activity in the absence of added ATP. Also, most recently, Conricode *et al.* [26] demonstrated that, in lung fibroblast membranes, regulation of PLD activity by PKC does not involve a protein-phosphorylation reaction.

If one accepts the idea that a protein-phosphorylation reaction is not involved in the regulation of PLD activity, then it becomes difficult to interpret the inhibitory effect of staurosporine on PMA-induced phospholipid hydrolysis, which appears to inhibit PKC activity through the catalytic site [11]. However, staurosporine was shown both to activate and to inhibit a number of different serine/threonine and tyrosine kinase activities [27,28]. Thus staurosporine is a considerably less specific inhibitor of PKC than is sphingosine. Because of these diverse effects of staurosporine, the present data cannot be taken as evidence that inhibition of PMA-induced PLD activity was directly due to the inhibition of PKC-mediated protein phosphorylation. Staurosporine has been shown to induce association of PKC with membranes [29]. This process, if it also exists in NIH 3T3 fibroblasts, could lead to increased down-regulation of a stimulatory PKC isoform in membranes. Alternatively, staurosporine may selectively promote translocation of an inhibitory PKC isoform, resulting in decreased stimulation of PLD activity by the stimulatory PKC isoform.

Although the above possibilities merit further investigation, on the basis of available data it is not possible to rule out completely the possibility that positive regulation of PLD activity by PKC involves a protein-phosphorylation reaction. Thus one cannot ignore the fact that under certain conditions okadaic acid enhanced, even if only slightly, PMA-induced phospholipid hydrolysis. Furthermore, in a previous study [30], we observed that addition of PMA to membranes isolated from *ras*- or *raf*-transformed fibroblasts enhanced phospholipid hydrolysis in an ATP- (and GTP)-dependent manner. Accordingly, it is possible that PKC can positively regulate PLD activity by both phosphorylation-dependent and -independent mechanisms, and that the inhibitory effect of sphingosine was due to the inhibition of the former process.

In contrast with the inhibitory effects of sphingosine, the stimulatory effect of sphingosine on PLD activity, as well as its potentiating effects on PMA-, bryostatin- and PDGF-stimulated phospholipid hydrolysis, occurred rapidly. Several observations, in this and a previous study, suggest that the stimulatory effects of sphingosine are composed of two different mechanisms. Thus we have reported previously [31] that the lipid-peroxidation

product 4-hydroxynonenal specifically inhibited the effect of sphingosine, but not that of PMA, on phospholipid hydrolysis. This indicates that sphingosine is likely to have a PKC-independent action on PLD activity (mechanism 2). This is supported by the present finding that the effect of sphingosine was only decreased, but not abolished, in fibroblasts in which PKC activity was down-regulated.

On the other hand, even maximally effective concentrations of 4-hydroxynonenal inhibited the stimulatory effect of sphingosine by only about 60% [31], which leaves open the possibility that sphingosine may also utilize a PKC-dependent mechanism to stimulate PLD activity (mechanism 3). Involvement of a PKC isoform in the stimulatory action of sphingosine is possible only if this isoform negatively regulates PLD activity, and if it is (at least) partially active in fibroblasts. The following evidence collectively suggests that this may be the case. (a) Staurosporine and down-regulation of PKC activity by PMA pretreatment enhanced similarly and in a non-additive manner the activity of PLD; these effects were also non-additive with that of sphingosine. (b) The protein phosphatase inhibitor okadaic acid [9,10] inhibited PLD activity in the control, but not in the PMA-pretreated cells, in which PKC activity was down-regulated.

Since PMA may well enhance the above inhibitory component, as indicated by the ability of okadaic acid to inhibit the overall stimulatory effect of PMA, potentiation of the stimulatory effects of PKC activators by sphingosine could be due to the inhibition of this inhibitory component. However, further work is required to prove finally that regulation of PLD activity by PKC activators indeed involves a negative regulatory component which is particularly sensitive to the inhibitory effect of sphingosine.

Work from several laboratories indicates that phosphatidic acid, the primary product of PLD action, is a mitogen in fibroblasts and other cells lines [32–38]. In addition, sphingosine potentiates the mitogenic effects of PMA and a number of growth factors [39,40], and it also enhances the effect of PMA on the formation of phosphatidic acid [40,41]. In fact, phosphatidic acid has been proposed to mediate the co-mitogenic effects of sphingosine [40]. On the basis of these data, it appears that negative regulation by a PKC isoform might serve to prevent extensive formation of phosphatidic acid through the action of PLD, and that the co-mitogenic effect of sphingosine may derive, at least partly, from its ability to release the phospholipid-hydrolysing system from such inhibition.

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REFERENCES

- Hannun, Y. A., Loomis, C. R., Merrill, A. H. & Bell, R. M. (1986) *J. Biol. Chem.* **261**, 12604–12609
- Hannun, Y. A. & Bell, R. M. (1989) *Science* **243**, 500–507
- Merrill, A. H. & Stevens, V. L. (1989) *Biochim. Biophys. Acta* **1010**, 131–139
- Kikkawa, V., Kishimoto, A. & Nishizuka, Y. (1989) *Annu. Rev. Biochem.* **58**, 31–44
- Kiss, Z. & Anderson, W. B. (1990) *J. Biol. Chem.* **265**, 7345–7350
- Lavie, Y. & Liscovitch, M. (1990) *J. Biol. Chem.* **265**, 3868–3872
- Rodriguez-Pena, A. & Rozengurt, E. (1984) *Biochem. Biophys. Res. Commun.* **120**, 1053–1059
- Hovis, J. G., Stumpo, D. J., Halsey, D. L. & Blackshear, P. J. (1986) *J. Biol. Chem.* **261**, 10380–10386
- Bialojan, C. & Takai, A. (1988) *Biochem. J.* **256**, 283–290
- Cohen, P. (1989) *Annu. Rev. Biochem.* **58**, 453–508
- Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. & Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 397–402
- Pettit, G. R., Herald, C. L., Doubek, D. L. & Herald, D. E. (1982) *J. Am. Chem. Soc.* **109**, 6846–6848
- Kiss, Z. (1991) *Lipids* **26**, 321–323
- Kiss, Z., Crilly, K. & Chattopadhyay, J. (1991) *Eur. J. Biochem.* **197**, 785–790
- Cook, S. J. & Wakelam, M. J. O. (1989) *Biochem. J.* **263**, 581–587
- Kiss, Z., Chattopadhyay, J. & Pettit, G. R. (1991) *Biochem. J.* **273**, 189–194
- Kiss, Z. & Anderson, W. B. (1989) *J. Biol. Chem.* **264**, 1483–1487
- Kiss, Z. (1976) *Eur. J. Biochem.* **67**, 557–561
- Okazaki, T., Bielawska, A., Bell, R. M. & Hannun, Y. A. (1990) *J. Biol. Chem.* **265**, 15823–15831
- Hennings, H., Blumberg, P. M., Pettit, G. R., Herald, C. L., Shores, R. & Yuspa, S. H. (1987) *Carcinogenesis* **8**, 1343–1346
- Kraft, A. S., Smith, J. B. & Berkow, R. L. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1334–1338
- Kiss, Z., Deli, E., Shoji, M., Koeffler, H. P., Pettit, G. R., Vogler, W. & Luo, J. F. (1987) *Cancer Res.* **47**, 1302–1307
- Kiss, Z., Deli, E., Girard, P., Pettit, G. R. & Kuo, J. F. (1987) *Biochem. Biophys. Res. Commun.* **146**, 208–215
- Kiss, Z. (1992) *Biochem. J.* **285**, 229–233
- Goldkorn, T., Dressler, K. A., Muindi, J., Radin, N. S., Mendelsohn, J., Menaldino, D., Liotta, D. & Kolesnick, R. N. (1991) *J. Biol. Chem.* **266**, 16092–16097
- Conricode, K. M., Brewer, K. A. & Exton, J. H. (1992) *J. Biol. Chem.* **267**, 7199–7202
- Kocher, J. & Clemetson, K. J. (1991) *Biochem. J.* **275**, 301–306
- Secrist, J. P., Sehgal, I., Powis, G. & Abraham, R. T. (1990) *J. Biol. Chem.* **265**, 20394–20400
- Wolf, M. & Baggiolini, M. (1988) *Biochem. Biophys. Res. Commun.* **154**, 1273–1279
- Kiss, Z., Rapp, U. R., Pettit, G. R. & Anderson, W. B. (1991) *Biochem. J.* **276**, 505–509
- Kiss, Z., Crilly, K. S., Rossi, M. A. & Anderson, W. B. (1992) *Biochim. Biophys. Acta* **1124**, 300–302
- Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J. & De Laat, S. W. (1986) *Nature (London)* **323**, 171–173
- Yu, C. L., Tsai, M. H. & Stacey, D. W. (1988) *Cell* **52**, 63–71
- Imagawa, W., Bandyopadhyay, G. K., Wallace, D. & Nandi, S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4122–4126
- van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T. & Moolenaar, W. H. (1989) *Cell* **59**, 45–54
- Knauss, T. C., Jaffer, F. E. & Abboud, H. E. (1990) *J. Biol. Chem.* **265**, 14457–14463
- Van Corven, E. J., Van Rijswijk, A., Jalink, K., Van Der Bend, R. L., Van Blitterswijk, W. J. & Moolenaar, W. H. (1992) *Biochem. J.* **281**, 163–169
- Fukami, K. & Takenawa, T. (1992) *J. Biol. Chem.* **267**, 10988–10993
- Zhang, H., Buckley, N. E., Gibson, K. & Spiegel, S. (1990) *J. Biol. Chem.* **265**, 76–81
- Zhang, H., Desai, N. N., Murphey, J. M. & Spiegel, S. (1990) *J. Biol. Chem.* **265**, 21309–21316
- Kiss, Z. (1990) *Prog. Lipid Res.* **29**, 141–166