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SPHINGOSINE-LIKE STIMULATORY EFFECTS OF PROPRANOLOL ON PHOSPHOLIPASE D ACTIVITY IN NIH 3T3 FIBROBLASTS

ZOLTAN KISS*
Hormel Institute, University of Minnesota, Austin, MN 55912, U.S.A.

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Abstract—Propranolol and sphingosine exhibit several common biochemical effects, including inhibition of phosphatidic acid phosphohydrolase and protein kinase C (PKC) activities. In NIH 3T3 fibroblasts, sphingosine has also been shown to stimulate phospholipase D (PLD)-mediated hydrolysis of both phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) (Kiss Z and Anderson WB, *J Biol Chem* **265**: 7345–7350, 1990). The present study demonstrates that in [^{14}C]palmitic acid-labeled NIH 3T3 fibroblasts, propranolol (50–100 μM) and sphingosine had similar stimulatory effects on PLD-mediated synthesis of phosphatidylethanol in the presence of ethanol. In [^{14}C]choline- and [^{14}C]-ethanolamine-labeled fibroblasts, both compounds also stimulated the hydrolysis of both [^{14}C]PtdCho and [^{14}C]PtdEtn. However, while sphingosine preferentially stimulated PtdEtn hydrolysis, propranolol had greater effects on PtdCho hydrolysis. At each time point examined (15–45 min), lower concentrations (25–50 μM) of propranolol and 100 nM phorbol 12-myristate 13-acetate (PMA) synergistically enhanced PtdEtn hydrolysis; a higher concentration (100 μM) of propranolol inhibited this PMA effect only when the incubation time was 45 min. On the other hand, propranolol (10–100 μM) had either no effect or it inhibited PMA-induced PtdCho hydrolysis after treatments for 15 or 45 min, respectively. These potentiating and inhibitory actions of propranolol on the hydrolysis of PtdCho and PtdEtn were similarly elicited by sphingosine. The present study identified the PLD system as another common target for the pharmacological actions of sphingosine and propranolol.

Key words: propranolol; sphingosine; phorbol ester; phospholipase D

Sphingoid bases, natural long-chain amino bases, can be considered as natural amphiphilic cations [1]. On this basis, one would expect that the biochemical actions of sphingosine would be similar to those elicited by other amphiphilic cations, such as propranolol and chlorpromazine. Indeed, propranolol and sphingosine have been shown to commonly inhibit the activity of a soluble form of phosphatidic acid phosphohydrolase [2–6], and that of PKC† [7, 8].

Recently, several laboratories reported that sphingosine can also stimulate PLD-mediated hydrolysis of phospholipids [9–12], whereas a similar effect of propranolol has not been demonstrated yet. Determination of the possible effects of propranolol on PLD activity is warranted for at least two reasons. First, a relatively large number of laboratories [see, for example, Refs. 13–20] have used propranolol as an inhibitor of phosphatidic acid phosphohydrolase to evaluate the physiological role of PLD. Second, a similar action of sphingosine and propranolol on the PLD system would provide further argument that sphingosine is indeed a natural amphiphilic cation.

PKC is perhaps the most important regulator of the PLD system identified thus far [reviewed in Ref. 21]. In NIH 3T3 fibroblasts, activators of PKC, including PMA and 1,2-diacylglycerol, were shown to stimulate the hydrolysis of both PtdCho and PtdEtn [22, 23]. Interestingly, while relatively low concentrations of sphingosine (5–20 μM) rapidly (3–15 min) stimulate PLD activity, inhibition of PMA-induced phospholipid hydrolysis requires higher concentrations of sphingosine (~50 μM) and longer incubation times (30–60 min) [11]. In addition, lower concentrations of sphingosine synergistically enhance the effect of PMA on PtdEtn hydrolysis, and at higher concentrations it preferentially inhibits PMA-induced PtdCho hydrolysis [11]. In view of the important regulatory role of PKC and the ability of propranolol to inhibit this enzyme, it was also of interest to determine how propranolol might affect PMA-induced phospholipid hydrolysis. In the present study it is shown that in NIH 3T3 fibroblasts propranolol mimicked the effects of sphingosine on both the unstimulated and PMA-stimulated PLD system with the exception that while sphingosine preferentially enhanced PtdEtn hydrolysis, propranolol had greater stimulatory effects on PtdCho hydrolysis.

MATERIALS AND METHODS

Materials. PMA, DL-propranolol, sphingosine,

* Correspondence: Dr. Zoltan Kiss, The Hormel Institute, University of Minnesota, 801 16th Avenue NE, Austin, MN 55912. Tel. (507) 437-9645; FAX (507) 437-9606.

† Abbreviations: PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; and PtdEtOH, phosphatidylethanol.

chlorpromazine, trifluoperazine and Dowex-50W (H^+ form) were purchased from the Sigma Chemical Co. (St. Louis, MO); [*methyl*-^{14}C]choline chloride (50 mCi/mmol), [2-^{14}C]ethanolamine (50 mCi/mmol), and [1-^{14}C]palmitic acid (60 mCi/mmol) were from Amersham (Arlington Heights, IL); tissue-culture reagents were bought from GIBCO (Grand Island, NY), PtdEtOH was purchased from Avanti Polar Lipids (Alabaster, AL).

Cell culture. NIH 3T3 clone-7 fibroblasts were cultured continuously in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, penicillin (50 U/mL)/streptomycin (50 μ/mL) and glutamine (2 mM). Fibroblasts were seeded in 150-mm-diameter plastic dishes, and growing (70–80% confluent) cell populations were harvested after 2 days in culture.

Treatment of fibroblasts labeled with [^{14}C]choline, [^{14}C]ethanolamine or [^{14}C]palmitic acid. Fibroblasts were grown in 150-mm-diameter dishes for 48 hr in the presence of [2-^{14}C]ethanolamine (0.25 μCi/mL) or [*methyl*-^{14}C]choline (0.30 μCi/mL), or for 24 hr in the presence of [1-^{14}C]palmitic acid (0.25 μCi/mL). Fibroblasts were washed and then incubated in fresh medium for 3 hr [to decrease the cellular level of unincorporated radiolabeled precursors; see Refs. 24–26]. Fibroblasts were harvested by gentle scraping from 3 to 6 dishes. Before treatments, scraped cells were incubated for 20 min (recovery period) to allow the slightly elevated 1,2-diacylglycerol level to return to the control value [27]. Then, washed fibroblasts (1.0 to 1.25×10^6 cells/mL) were incubated (final vol. 0.25 mL) at 37° in the presence of agents as indicated. In the case of [^{14}C]choline- and [^{14}C]ethanolamine-labeled fibroblasts, the incubation medium contained either 20 mM unlabeled choline or 2 mM unlabeled ethanolamine, respectively, to prevent metabolism of newly formed [^{14}C]choline and [^{14}C]ethanolamine [22, 25]. Incubations were terminated by the addition of 4 mL of chloroform : methanol (1 : 1, v/v).

Separation of ^{14}C-labeled hydrolytic products. PtdEtOH was separated from other phospholipids on potassium oxalate (1%)-impregnated silica gel H plates (Analtech) by using the solvent system of chloroform : methanol : acetone : acetic acid : water (50 : 10 : 15 : 10 : 2, by vol.). The choline and ethanolamine metabolites were fractionated on Dowex-50-W(H^+)-packed columns (Bio-Rad Econo columns; 1-mL bed vol.) as described by Cook and Wakelam [28] with the modifications described previously [11]. The metabolites of [^{14}C]choline and [^{14}C]ethanolamine were identified further by thin-layer chromatography [22]. Contamination of the [^{14}C]ethanolamine fraction by [^{14}C] choline was less than 1%.

Experiments with attached fibroblasts. As discussed in detail elsewhere [26], scraped cells retained sensitivity to PMA, sphingosine and hormones with respect to the stimulation of PLD activity. On the other hand, scraped cells contain much lower levels of background [^{14}C]ethanolamine and [^{14}C]choline [25, 26], which significantly increase the sensitivity of the assay of PLD activity. Thus, throughout this study, most experiments were performed with labeled suspended fibroblasts. However, the effects observed with maximally effective concentrations of propranolol (usually 100 μM) were also verified in attached cells grown and labeled in 6-well tissue culture dishes.

Table 1. Comparison of the effects of propranolol, sphingosine and PMA on the formation of PtdEtOH in [^{14}C]palmitate-labeled NIH 3T3 fibroblasts*

Addition	Formation of [^{14}C]PtdEtOH (dpm/10^6 cells/20 min)
None	570 ± 180
Propranolol, 50 μM	1240 ± 160
Propranolol, 100 μM	2990 ± 310
Propranolol, 250 μM	1470 ± 380
Sphingosine, 30 μM	4070 ± 490
PMA, 100 nM	8310 ± 670

* NIH 3T3 fibroblasts were prelabeled with [^{14}C]palmitic acid, and then suspended labeled fibroblasts were incubated for 20 min as described in Materials and Methods. The ^{14}C contents of PtdCho and PtdEtn were 730,000 and 204,000 dpm/10^6 cells, respectively. Data are the means ± SEM of three incubations. This experiment was repeated once with similar results.

RESULTS

Comparison of the effects of propranolol, sphingosine and PMA on the formation of PtdEtOH. Formation of metabolically stable PtdEtOH from phospholipids and ethanol is an accepted measure of PLD activity. As shown in Table 1, addition of 50 and 100 μM propranolol to [^{14}C]palmitic acid-labeled fibroblasts in the presence of ethanol for 20 min enhanced [^{14}C]PtdEtOH formation about 2.2- and 5.2-fold, respectively. In comparison, maximally effective concentrations of sphingosine (30 μM) and PMA (100 nM) stimulated PtdEtOH formation about 7.1- and 14.6-fold, respectively. These data establish that in NIH 3T3 fibroblasts propranolol stimulates PtdEtOH formation, although somewhat less effectively than sphingosine.

In another experiment, we treated the [^{14}C]-palmitate-labeled fibroblasts with propranolol (100 μM), sphingosine (30 μM), or PMA (100 nM) in the absence of ethanol for 20 min to determine their effects on the formation of [^{14}C]1,2-diacylglycerol. While PMA enhanced the formation of [^{14}C]1,2-diacylglycerol about 1.6-fold, neither propranolol nor sphingosine had detectable stimulatory effects (Table 2).

Comparison of the effects of propranolol and sphingosine on the hydrolysis of PtdCho and PtdEtn. We established earlier that in PMA- and sphingosine-stimulated labeled fibroblasts increased formation of [^{14}C]choline and [^{14}C]ethanolamine was entirely due to PLD-mediated hydrolysis of the corresponding prelabeled phospholipids [9, 22]. Therefore, if propranolol also increases the formation of [^{14}C]-choline and [^{14}C]ethanolamine through the stimulation of PLD, then propranolol should not be able to further increase the maximal stimulatory effects

Table 2. Comparison of the effects of PMA, propranolol and sphingosine on the formation of 1,2-diacylglycerol in [^{14}C]palmitate-labeled NIH 3T3 fibroblasts*

Addition	Formation of [^{14}C]1,2-diacylglycerol (dpm/10^6 cells/20 min)
None	2630 ± 290
PMA, 100 nM	4280 ± 370
Propranolol, 100 μM	2990 ± 410
Sphingosine, 30 μM	2510 ± 220

* NIH 3T3 fibroblasts were prelabeled with [^{14}C]palmitic acid, and then suspended labeled fibroblasts were treated with the above agents for 20 min. Data are the means ± SEM of four incubations. Similar results were obtained in two other experiments.

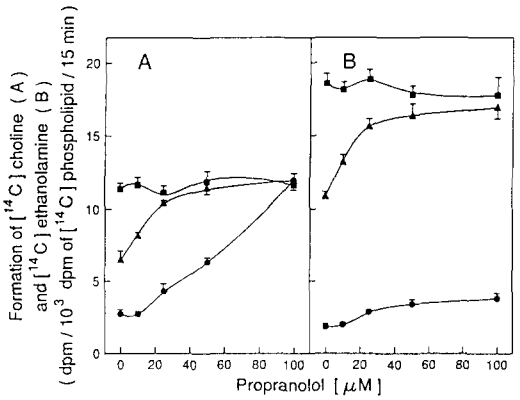

Fig. 1. Combined effects of propranolol and sphingosine on the hydrolysis of PtdCho and PtdEtn in NIH 3T3 fibroblasts. Attached fibroblasts were labeled with [^{14}C]-choline (A) or [^{14}C]ethanolamine (B) for 48 hr, and then suspended fibroblasts were treated with various concentrations of propranolol (10–100 μM) in the absence (●) or presence of 15 μM sphingosine (▲) or 30 μM sphingosine (■) for 15 min. The ^{14}C content of PtdCho and PtdEtn in this experiment was 911,000 and 1.28 10^6 dpm/10^6 cells, respectively, and showed little variation (±7%) in the following experiments. Each point is the mean ± SEM of three incubations. Similar results were obtained in two other experiments.

of sphingosine. The next experiment was designed to test this possibility.

As shown in Fig. 1A, both propranolol and sphingosine significantly stimulated PtdCho hydrolysis. Maximally effective concentrations of sphingosine (30 μM) and propranolol (100 μM) were equipotent and failed to enhance each other's effects. On the other hand, the effect of 15 μM sphingosine, which exerted a half-maximal stimulatory effect on PtdCho hydrolysis, was synergistically enhanced by 10–25 μM concentrations of propranolol. However, these latter combined effects did not exceed the stimulatory effect of 30 μM sphingosine alone.

Propranolol had significantly smaller, while sphingosine had significantly greater effects on PtdEtn hydrolysis (Fig. 1B), compared with their effects on PtdCho hydrolysis (Fig. 1A). However, lower concentrations of propranolol (10–25 μM) and 15 μM sphingosine again synergistically enhanced PtdEtn hydrolysis, whereas the effect of 30 μM sphingosine was not altered by any concentration of propranolol (Fig. 1B).

Treatment of [^{14}C]choline-labeled fibroblasts with propranolol (100 μM) or sphingosine (30 μM) up to 45 min failed to enhance the formation of [^{14}C]-choline phosphate (data not shown). However, treatment of [^{14}C]ethanolamine-labeled fibroblasts with these compounds for 45 min slightly (~1.3- to 1.5-fold) elevated the formation of [^{14}C]ethanolamine phosphate. This may indicate that sphingosine and propranolol, similar to ethanol [29], have small stimulatory effects on a PtdEtn-specific phospholipase C. However, under the conditions used, increased formation of [^{14}C]ethanolamine and [^{14}C]ethanolamine phosphate occurs by independent mechanisms [29].

Combined effects of propranolol and PMA on the hydrolysis of PtdCho and PtdEtn. In a previous study [11], sphingosine and PMA synergistically enhanced PtdEtn hydrolysis after a 15-min incubation, while higher concentrations (30–50 μM) of sphingosine inhibited PMA-induced phospholipid hydrolysis only after a longer (45 min) incubation period. Presently, at the shorter incubation time (15 min), 10–100 μM concentrations of propranolol had no significant inhibitory effects on PMA-induced PtdCho hydrolysis (Fig. 2A), whereas 25 and 50 μM concentrations of propranolol enhanced the stimulatory effect of PMA on PtdEtn hydrolysis (Fig. 2B). After a longer incubation period (45 min), 25–100 μM concentrations of propranolol effectively inhibited PMA-induced PtdCho hydrolysis (Fig. 3A). On the other hand, only 100 μM propranolol inhibited, and only slightly (by 19%), PMA-induced PtdEtn hydrolysis (Fig. 3B).

Synergistic stimulation of PtdEtn hydrolysis by propranolol and PMA may be an indication that both agents acted through the PKC system. However, while the stimulatory effect of PMA on PtdEtn hydrolysis was shown to be inhibited by the PKC inhibitors staurosporine and 1-(5-isoquinoli-

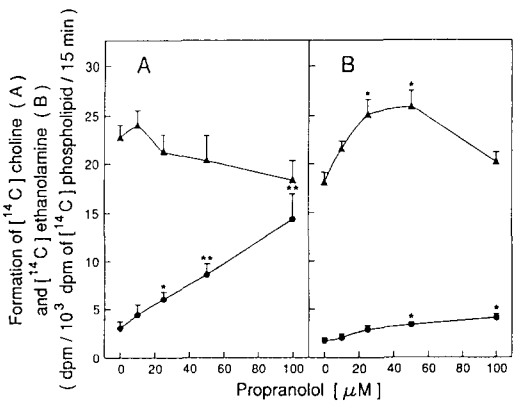

Fig. 2. Short-term combined effects of propranolol and PMA on the hydrolysis of PtdCho and PtdEtn in NIH 3T3 fibroblasts. Fibroblasts were labeled with [^{14}C]choline (A) or [^{14}C]ethanolamine (B) for 48 hr, and then suspended fibroblasts were treated with 10–100 μM concentrations of propranolol in the absence (●) or presence of 100 nM PMA (▲) for 15 min. Each point is the mean ± SEM of eight independent incubations performed on the same day. Similar results were obtained in two other experiments (performed in triplicate), except that propranolol was a somewhat more effective inhibitor of PtdCho hydrolysis. Key: (*,**) significantly ($P < 0.05^* - 0.01^{**}$) different from the respective control.

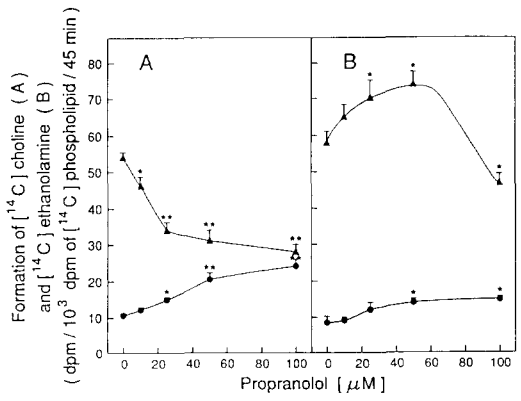

Fig. 3. Longer-term combined effects of propranolol and PMA on the hydrolysis of PtdCho and PtdEtn in NIH 3T3 fibroblasts. Fibroblasts were labeled with [^{14}C]choline (A) or [^{14}C]ethanolamine (B) for 48 hr, and then suspended fibroblasts were treated with 10–100 μM concentrations of propranolol in the absence (●) or presence of 100 nM PMA (▲) for 45 min. Each point is the mean ± SEM of eight independent incubations performed on the same day. Similar results were obtained in three other experiments performed in triplicate. Key: (*,**) significantly ($P < 0.05^* - 0.01^{**}$) different from the respective control.

nylsulfonyl)-2-methylpiperazine [29], these inhibitors failed to modify the stimulatory effects of propranolol on the hydrolysis of either PtdEtn or PtdCho (data not shown).

Stimulatory effects of propranolol on PLD activity in fibroblasts after chronic treatment with PMA. We have shown earlier [11] that prolonged (24 hr) treatment of fibroblasts with PMA, which down-regulates PKC activity [discussed in Ref. 11], also diminishes the stimulatory effect of newly added PMA on PLD activity. To examine if PKC played a role in the mediation of propranolol effects, we determined propranolol-induced phospholipid hydrolysis in fibroblasts chronically treated with PMA. In agreement with our previous observations [11, 23], chronic PMA treatment enhanced the hydrolysis of both PtdCho and PtdEtn, whereas newly added PMA had no stimulatory effect in the pretreated cells (Table 3). Importantly, the stimulatory effects of propranolol in PMA-treated cells were inhibited only slightly (PtdCho hydrolysis) or were not inhibited at all (PtdEtn hydrolysis) (Table 3). These data further suggest that stimulation of phospholipid hydrolysis by propranolol does not involve the PKC system.

Effects of other amphiphilic cations on phospholipid hydrolysis. It is reasonable to assume that propranolol is not the only amphiphilic cation that can stimulate PLD activity. To verify this possibility, the effects of chlorpromazine and trifluoperazine on PLD activity were also examined. Both compounds had propranolol-like stimulatory effects on the hydrolysis of PtdEtn and PtdCho except that they were maximally effective at a 50 μM concentration. Similarly, both chlorpromazine and trifluoperazine were somewhat more effective than propranolol in inhibiting PMA-induced hydrolysis of phospholipids (data not shown).

DISCUSSION

The present study identified the PLD system as an additional common target for the pharmacological actions of propranolol and sphingosine in fibroblasts. Both compounds enhanced the synthesis of PtdEtOH, a marker of PLD activity, as well as PLD-mediated hydrolysis of PtdCho and PtdEtn. The effects of a maximally effective concentration (30 μM) of sphingosine on PLD activity were not altered by propranolol. In addition, at higher concentrations, both sphingosine [11] and propranolol (this paper) preferentially inhibited PMA-induced PtdCho hydrolysis, whereas at lower concentrations both compounds preferentially enhanced the stimulatory effect of PMA on PtdEtn hydrolysis. These results suggest that stimulation of PLD-mediated hydrolysis of phospholipids by sphingosine and propranolol involves similar mechanisms.

Although the PLD system is clearly a common target for the actions of propranolol and sphingosine, their relative potencies on the hydrolysis of PtdCho and PtdEtn are different. Thus, while propranolol preferentially stimulated the hydrolysis of PtdCho, sphingosine had significantly greater stimulatory effects on PtdEtn hydrolysis. As a result, maximally

Table 3. Comparison of the effects of propranolol and PMA on the hydrolysis of PtdCho and PtdEtn in untreated and PMA-treated NIH 3T3 fibroblasts*

Addition	Formation of: [^{14}C]Choline (dpm/10^6 dpm of [^{14}C]phospholipid/20 min)		[^{14}C]Ethanolamine (dpm/10^6 dpm of [^{14}C]phospholipid/20 min)	
	Untreated	PMA-treated	Untreated	PMA-treated
None	2210 ± 50	4130 ± 430	2450 ± 280	6390 ± 340
Propranolol, 50 μM	6670 ± 330	6690 ± 500	4190 ± 160	8080 ± 480
Propranolol, 100 μM	10,880 ± 230	10,820 ± 620	5600 ± 270	9260 ± 190
PMA, 100 nM	13,490 ± 1110	4220 ± 260	14,450 ± 960	6170 ± 400

* Fibroblasts were labeled with [^{14}C]choline or [^{14}C]ethanolamine for 48 hr as described in Materials and Methods. Where indicated, 400 nM PMA was added for the last 24 hr of the labeling period (PMA-treated). Suspended fibroblasts were incubated in the absence and presence of propranolol or PMA as indicated. Data are the means ± SEM of four incubations. Similar results were obtained in two other experiments.

effective concentrations of sphingosine and propranolol were equipotent in stimulating PtdCho hydrolysis, whereas sphingosine was about 5 times as potent as propranolol in stimulating PtdEtn hydrolysis. The reason for the observed differences in the relative potencies of propranolol and sphingosine is unknown. We are presently investigating the possibility that the hydrolysis of PtdCho and PtdEtn is catalyzed by separate PLD activities, perhaps in different cell compartments, which are differentially sensitive to the actions of sphingosine and propranolol. In this context, it is important to mention that most tissues examined were found to express two different PLD activities. A membrane-bound PLD appeared to specifically hydrolyze PtdCho, while a cytoplasmic enzyme preferentially hydrolyzed PtdEtn [30].

Because the effect of propranolol on PLD activity has gone undetected in so many studies [13–20], it is possible that the presently described effects of propranolol occur only in certain cell types. However, it also should be noted that propranolol stimulates PLD activity only at relatively lower concentrations (up to 100 μM concentration), while for the inhibition of phosphatidic acid phosphohydrolase propranolol is most often used at 200–300 μM concentrations. While this paper was in preparation, Singh *et al.* [31] reported that certain amphiphilic cations (propranolol was not studied), including mepacrine, desipramine and chlorpromazine, stimulate PLD activity in the human neuroblastoma cell line LA-N-2. These data, combined with ours on the effects of trifluoperazine, suggest that stimulation of PLD activity is elicited by most, if not all, amphiphilic cations, and that this phenomenon is not restricted to fibroblasts.

In summary, the present study demonstrates that in NIH 3T3 fibroblasts propranolol and sphingosine can similarly stimulate PLD activity. While these data provide an additional argument for sphingosine being an endogenous amphiphilic cation [1], they also indicate that, at least in certain cell types, propranolol cannot be used for specific inhibition of phosphatidic acid phosphohydrolase.

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REFERENCES

1. Liscovitch M and Lavie Y, Sphingoid bases as endogenous cationic amphiphilic "drugs." *Biochem Pharmacol* **42**: 2071–2075, 1991.
2. Pappu AS and Hauser G, Propranolol-induced inhibition of rat brain cytoplasmic phosphatidate phosphohydrolase. *Neurochem Res* **8**: 1565–1575, 1983.
3. Koul O and Hauser G, Modulation of rat brain cytosolic phosphatidate phosphohydrolase: Effect of cationic amphiphilic drugs and divalent cations. *Arch Biochem Biophys* **253**: 453–461, 1987.
4. Gomez-Muñoz A, Hamza EH and Brindley DN, Effects of sphingosine, albumin and unsaturated fatty acids on the activation and translocation of phosphatidate phosphohydrolases in rat hepatocytes. *Biochim Biophys Acta* **1127**: 49–56, 1992.
5. Lavie Y, Piterman O and Liscovitch M, Inhibition of phosphatidic acid phosphohydrolase activity by sphingosine. Dual action of sphingosine in diacylglycerol signal termination. *FEBS Lett* **277**: 7–10, 1990.
6. Mullmann TJ, Siegel MI, Egan RW and Billah MM, Sphingosine inhibits phosphatidate phosphohydrolase in human neutrophils by a protein kinase C-independent mechanism. *J Biol Chem* **266**: 2013–2016, 1991.
7. Sozzani S, Agwu DE, McCall CE, O'Flaherty JT, Schmitt JD, Kent JD and McPhail LC, Propranolol, a phosphatidate phosphohydrolase inhibitor, also inhibits protein kinase C. *J Biol Chem* **267**: 20481–20488, 1992.
8. Hannun YA, Loomis CR, Merrill AH Jr and Bell RM, Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding *in vitro* and in human platelets. *J Biol Chem* **261**: 12604–12609, 1986.
9. Kiss Z and Anderson WB, ATP stimulates the hydrolysis of phosphatidylethanolamine in NIH 3T3 cells. *J Biol Chem* **265**: 7345–7350, 1990.
10. Lavie Y and Liscovitch M, Activation of phospholipase

D by sphingoid bases in NG108–15 neural-derived cells. *J Biol Chem* **265**: 3868–3872, 1990.
11. Kiss Z and Deli E, Regulation of phospholipase D by sphingosine involves both protein kinase C-dependent and -independent mechanisms in NIH 3T3 fibroblasts. *Biochem J* **288**: 853–858, 1992.
12. Desai NN, Zhang H, Olivera A, Mattie ME and Spiegel S, Sphingosine-1-phosphate, a metabolite of sphingosine, increases phosphatidic acid levels by phospholipase D activation. *J Biol Chem* **267**: 23122–23128, 1992.
13. Cabot MC, Welsh CJ, Cao HT and Chabbott H, The phosphatidylcholine pathway of diacylglycerol formation stimulated by phorbol diesters occurs via phospholipase D activation. *FEBS Lett* **233**: 153–157, 1988.
14. Liscovitch M and Amsterdam A, Gonadotropin-releasing hormone activates phospholipase D in ovarian granulosa cells. *J Biol Chem* **264**: 11762–11767, 1989.
15. Billah MM, Eckel S, Mullmann TJ, Egan RW and Siegel MI, Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidate and diglyceride levels in chemotactic peptide-stimulated human neutrophils. *J Biol Chem* **264**: 17069–17077, 1989.
16. Martinson EA, Trilivas I and Brown JH, Rapid protein kinase C-dependent activation of phospholipase D leads to delayed 1,2-diglyceride accumulation. *J Biol Chem* **265**: 22282–22287, 1990.
17. Song J, Pfeffer LM and Foster DA, v-Src increases diacylglycerol levels via a type D phospholipase-mediated hydrolysis of phosphatidylcholine. *Mol Cell Biol* **11**: 4903–4908, 1991.
18. Chabot MC, McPhail LC, Wykle RL, Kennerly DA and McCall CE, Comparison of diglyceride production from choline-containing phosphoglycerides in human neutrophils stimulated with *N*-formylmethionyl-leucylphenylalanine, ionophore A23187 or phorbol 12-myristate 13-acetate. *Biochem J* **286**: 693–699, 1992.
19. Purkiss JR and Boarder MR, Stimulation of phosphatidate synthesis in endothelial cells in response to P_2-receptor activation: Evidence for phospholipase C and phospholipase D involvement, phosphatidate and diacylglycerol interconversion and the role of protein kinase C. *Biochem J* **287**: 31–36, 1992.
20. Perry DK, Hand WL, Edmondson DE and Lambeth JD, Role of phospholipase D-derived diradylglycerol in the activation of the human neutrophil respiratory burst oxidase. Inhibition by phosphatidic acid phosphohydrolase inhibitors. *J Immunol* **149**: 2749–2758, 1992.
21. Kiss Z, Effects of phorbol ester on phospholipid metabolism. *Prog Lipid Res* **19**: 141–166, 1991.
22. Kiss Z and Anderson WB, Phorbol-ester stimulates hydrolysis of phosphatidylethanolamine in leukemic HL60, NIH 3T3 and baby hamster kidney cells. *J Biol Chem* **264**: 1483–1487, 1989.
23. Kiss Z and Garamszegi N, Protein kinase C-dependent stimulation of phospholipase D in phospholipase C-treated fibroblasts. *Lipids* **28**: 479–481, 1993.
24. Kiss Z, Chattopadhyay J and Pettit GR, Stimulation of phosphatidylcholine synthesis by activators of protein kinase C is dissociable from increased phospholipid hydrolysis. *Biochem J* **273**: 189–194, 1991.
25. Kiss Z, Determination of phospholipase D-mediated hydrolysis of phosphatidylethanolamine. *Lipids* **26**: 321–323, 1991.
26. Kiss Z, Differential effects of platelet-derived growth factor, serum and bombesin on phospholipase D-mediated hydrolysis of phosphatidylethanolamine in NIH 3T3 fibroblasts. *Biochem J* **285**: 229–233, 1992.
27. Kiss Z, Crilly K and Chattopadhyay J, Ethanol potentiates the stimulatory effects of phorbol ester, sphingosine and 4-hydroxynonenal on the hydrolysis of phosphatidylethanolamine in NIH 3T3 cells. *Eur J Biochem* **197**: 785–790, 1991.
28. Cook SJ and Wakelam MJO, Analysis of the water-soluble products of phosphatidylcholine breakdown by ion-exchange chromatography: Bombesin and TPA (12-*O*-tetradecanoylphorbol 13-acetate) stimulate choline generation in Swiss 3T3 cells by a common mechanism. *Biochem J* **263**: 581–587, 1989.
29. Kiss Z, The long-term combined stimulatory effects of ethanol and phorbol ester on phosphatidylethanolamine hydrolysis are mediated by a phospholipase C and prevented by overexpressed α-protein kinase C in fibroblasts. *Eur J Biochem* **209**: 467–473, 1992.
30. Wang P, Anthes JC, Siegel MI, Egan RW and Billah MM, Existence of cytosolic phospholipase D: Identification and comparison with membrane-bound enzyme. *J Biol Chem* **266**: 14877–14880, 1991.
31. Singh IN, Massarelli R and Kanfer JN, Activation of phospholipases D and A by amphiphilic cations of cultured LA-N-2 cells is G protein- and protein kinase C-independent. *J Lipid Med* **7**: 85–96, 1993.