Preferential inhibition of phorbol ester-induced hydrolysis of phosphatidylethanolamine by N-acetylsphingosine in NIH 3T3 fibroblasts

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Abstract It has been reported that in rat fibroblasts cell-permeable ceramide analogs inhibit agonist-induced phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine (PtdCho). Here we demonstrate that relatively short (30 min) treatments of NIH 3T3 fibroblasts with 15–60 μM concentrations of N-acetylsphingosine result in preferential, although not exclusive, inhibition of phorbol 12-myristate 13-acetate-induced PLD-mediated hydrolysis of phosphatidylethanolamine (PtdEtn). The results suggest that in different cell types the PtdEtn- and PtdCho-hydrolyzing PLD activities are differentially sensitive to the inhibitory effect of ceramide.

Key words: N-Acetylsphingosine; Phospholipase D; Phosphatidylethanolamine; Phosphatidylcholine; NIH 3T3 fibroblast

1. Introduction

It has recently been reported [1] that in rat fibroblasts the cell-permeable ceramide analog N-acetylsphingosine (C$_2$-ceramide) can inhibit phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine (PtdCho). Since this report appeared, several other laboratories also described, using other cell lines, inhibition of agonist-stimulated PLD activity by ceramide, except that in these latter studies the nature of phospholipid substrate was not identified [2–4].

In NIH 3T3 fibroblasts, activation of the PLD system(s) by the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) [5], sphingosine [6], nucleotides [6] and several hormones [7] were found to result in significant hydrolysis of both PtdCho and phosphatidylethanolamine (PtdEtn). Nevertheless, in many respects the regulation of PtdCho and PtdEtn hydrolysis in fibroblasts appears to be different, suggesting that these phospholipids may play different roles in signal transduction (reviewed in [8,9]). Importantly, PLD-mediated hydrolysis of PtdEtn has also been observed in several other cell lines [10–15], and in some cases only PtdEtn was hydrolyzed by activated PLD [16]. For these reasons, it may become important to identify the substrate(s) of the ceramide-inhibitable PLD system(s). Here we report that in NIH 3T3 fibroblasts C$_2$-ceramide preferentially, although not exclusively, inhibits PMA-induced PtdEtn hydrolysis.

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2. Materials and methods

2.1. Materials

C$_2$-ceramide was purchased from Matreya Inc.; it was delivered to fibroblasts in a 1:1 complex with fatty acid-free bovine serum albumin. PMA, SMase (from *Staphylococcus aureus*), and Dowex-50W[H$^+$ form] were from Sigma; [2-^{14}C]ethanolamine (60 mCi/mmol), [methyl-^{14}C]choline chloride (55 mCi/mmol), and [1-^{14}C]palmitic acid (60 mCi/ mmol) were from Amersham; tissue culture reagents were from Gibco BRL. Phosphatidylethanol (PtdEtOH) was from Avanti Polar Lipids Inc.

2.2. Cell culture

NIH 3T3 clone 7 fibroblasts were obtained from Dr. Douglas R. Lowy (National Cancer Institute, Bethesda, MD) and were cultured continuously in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) fetal calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml) and glutamine (2 mM).

2.3. Measurement of formation of PtdEtOH in NIH 3T3 fibroblasts prelabeled with [^{14}C]palmitic acid

Fibroblasts were grown in 6-well tissue culture dishes for 48 h to about 90% confluency, and labeled with [1-^{14}C]palmitic acid (0.35 μCi/ ml) for the last 24 h. The attached fibroblasts were washed, incubated in fresh Dulbecco's medium for an additional 2 h (to minimize the amount of unesterified labeled palmitic acid), and then treated with 100 nM PMA and/or 60 μM C$_2$-ceramide, or SMase (200 mU/ml), in the presence of 200 mM ethanol as indicated in Fig. 1. Incubations were performed in an incubator at 37°C and were terminated by scraping the fibroblasts into 2 ml of ice-cold methanol followed by rapid transfer of the methanol extract to 2 ml of chloroform. 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).

2.4. Determination of water-soluble products of phospholipid hydrolysis in NIH 3T3 fibroblasts

Fibroblasts, seeded (2 × 10^5/dish) in 150 mm-diameter plastic dishes, were incubated with either [methyl-^{14}C]choline (0.35 μCi/ml) or [2-^{14}C]ethanolamine (0.25 μCi/ml) for 60 h, washed, then incubated in fresh medium for 3.5 h (to decrease the cellular level of water-soluble ^{14}C-labeled precursors [17]. Fibroblasts were harvested by scraping from 3–6 dishes. After an additional 20 min incubation period (required for the slightly increased 1,2-diacylglycerol level to return to normal level [18]), fibroblasts were pelleted for 8 min at 500 × g. Resuspended fibroblasts were incubated (final vol. 0.25 ml; 1.8–2.2 × 10^6 cells/ml) in the presence of 2 mM ethanolamine or 20 mM choline, as appropriate, to prevent phosphorylation of newly formed [^{14}C]ethanolamine and [^{14}C]choline [5]. Incubations were terminated by addition of 4 ml of chloroform/methanol (1:1, v/v). Separation of ethanolamine and choline metabolites was performed on Dowex-50(H$^+$)-packed columns as described earlier [17,19]. Phospholipids were separated as in [20].

3. Results and discussion

In a previous study [19], we determined that treatments of NIH 3T3 fibroblasts with 200–400 mU/ml of bacterial SMase for 20–30 min was sufficient to degrade the entire cell surface pool of sphingomyelin (about 50% of the total cellular pool of
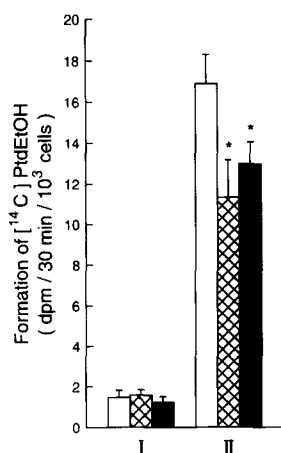
Fig. 1. Effects of SMase and C_2-ceramide on the formation of [^{14}C]PtdEtOH in [^{14}C]palmitic acid-labeled NIH 3T3 fibroblasts. NIH 3T3 fibroblasts, prelabeled with [^{14}C]palmitic acid for 24 h as described in section 2, were incubated for 30 min in the absence (I) or presence (II) of 100 nM PMA with the incubation medium containing no other addition (□) or containing 200 mU/ml of SMase (▨) or 60 μM C_2-ceramide (■); SMase and C_2-ceramide were added to the medium 5 min prior to the addition of PMA. Data are the mean ± S.E.M. of three incubations. Similar results were obtained in another experiment.

sphingomyelin) and that during this process only ceramide, but not sphingosine, was formed. We also found that at a density of 2×10^6 cells/ml, the highest concentration of C_2-ceramide with no significant cytotoxic effects over a 30-min incubation period was 60 μM. Thus, first we examined the effects of 200 mU/ml of SMase and 60 μM C_2-ceramide on the synthesis of PtdEtOH, which is a specific function of PLD [21,22], in [^{14}C]palmitic acid-labeled NIH 3T3 fibroblasts during a 30-min incubation period. In the absence of PMA, cells synthesized only small amounts of [^{14}C]PtdEtOH; this was not affected by either SMase or C_2-ceramide (Fig. 1; I). Addition of 100 nM PMA greatly enhanced the formation of [^{14}C]PtdEtOH; this PMA effect was similarly (23–33%) inhibited by SMase and C_2-ceramide (Fig. 1, II). Although these data confirmed previous data [1–4] that ceramide can inhibit PMA-induced PLD activity, under the present conditions the effect of ceramide was clearly less than that reported by others using different cell types and different incubation procedures.

In [^{14}C]palmitic acid labeled fibroblasts the ratio of ^{14}C activity in PtdCho and PtdEtn was 3.6:1. Preferential labeling of PtdCho if accompanied by only small effects of ceramide on PtdCho hydrolysis could clearly mask a major effect of ceramide on PtdEtn hydrolysis. Therefore, characterization of the actions of ceramide required separate analysis of PtdCho and PtdEtn hydrolysis. Previously, we determined that in [^{14}C]choline- and [^{14}C]ethanolamine-labeled NIH 3T3 fibroblasts the activators of PKC and several other agents as well stimulate the formation of [^{14}C]choline and [^{14}C]ethanolamine through PLD-mediated hydrolysis of the respective labeled cellular phospholipids [5–7]. Thus, in the following experiments we examined the effects of the cell-permeable ceramide analog, C_2-ceramide, on PLD-mediated hydrolysis of PtdCho and PtdEtn in [^{14}C]choline- and [^{14}C]ethanolamine-labeled fibroblasts, respectively. Only C_2-ceramide was used because com-

mercial SMase preparations contained significant phospholipase C activity, making interpretation of data difficult.

Treatment of [^{14}C]ethanolamine-labeled fibroblasts for 30 min with increasing concentrations of C_2-ceramide resulted in progressive inhibition of PMA-induced [^{14}C]PtdEtn hydrolysis; at the highest concentration (60 μM) tested, C_2-ceramide caused 55% inhibition of this PMA effect (Fig. 2A). In the absence of PMA, C_2-ceramide had no effect on the formation of [^{14}C]ethanolamine (Fig. 2A). Surprisingly, treatment of [^{14}C]choline-labeled fibroblasts with even 60 μM C_2-ceramide inhibited PMA-induced PtdCho hydrolysis only by 27% (Fig. 2B).

Recent evidence strongly indicate that the stimulatory effects of PMA on the hydrolysis of both PtdCho [4,23–25] and PtdEtn [16] are mediated by PKC-α. Furthermore, ceramide has been shown to inhibit PLD activity by preventing translocation of PKC-α to the membrane fraction [4]. Therefore, the observed differences in ceramide effects on the hydrolysis of PtdCho and PtdEtn can occur only if regulation of these hydrolytic processes by PKC-α involves, at least in part, different mechanisms. Since PKC is known to regulate PtdCho hydrolysis by both phosphorylation-dependent [26] and -independent [27] mechanisms, it seemed possible that these mechanisms play different roles in the regulation of PtdCho and PtdEtn hydrolysis. To examine this possibility, we determined the effects of bisindolylmaleimide GF 109203X, a potent and selective inhibitor of PKC-mediated protein phosphorylation [28], on the hydrolysis of these phospholipids. Surprisingly, even very high concentrations (25–75 μM) of GF 109203X inhibited PMA-induced PtdCho hydrolysis only by about 50% when the incubation time was 30 min (Fig. 3A). In contrast, these concentrations of GF 109203X almost completely inhibited PMA-induced PtdEtn hydrolysis (Fig. 3B). These data suggest that at this time point, the action of PMA on PtdCho hydrolysis
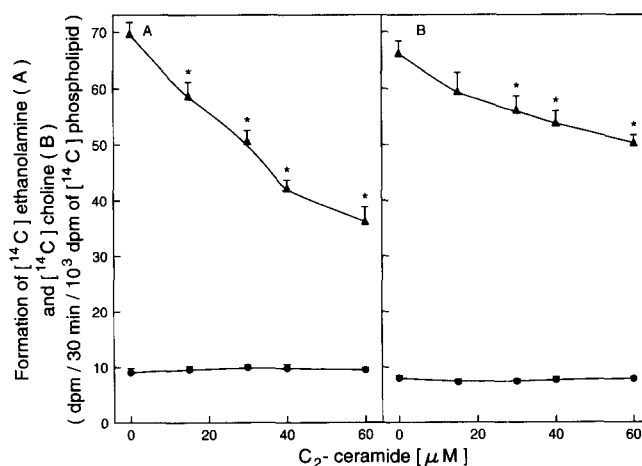

Fig. 2. Concentration-dependent effects of C_2-ceramide on the hydrolysis of PtdEtn and PtdCho in NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were labeled with [^{14}C]ethanolamine (A) or [^{14}C]choline (B) for 60 h, followed by treatment of labeled fibroblasts for 30 min with different concentrations of C_2-ceramide (15–60 μM) in the absence (●) or presence (▲) of 100 nM PMA. C_2-ceramide was added to the medium 5 min prior to the addition of PMA. Each point represents the mean ± S.E.M. of six independent incubations. Similar results were obtained in three other experiments (each performed in triplicate) using 20 min incubation time. *Significantly ($P < 0.01$) different from the value obtained with PMA alone.
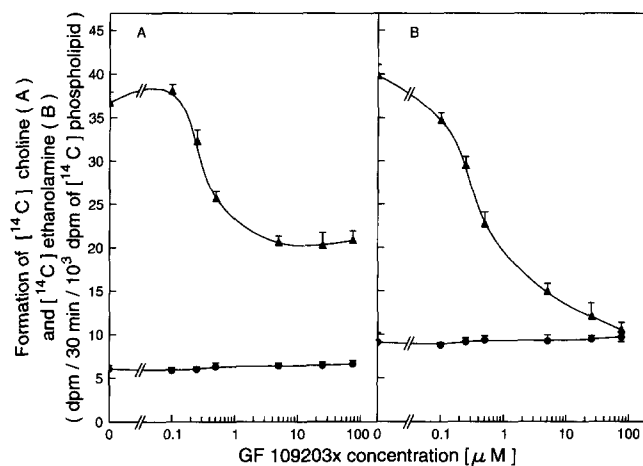
Fig. 3. Inhibition of PMA-induced hydrolysis of [^{14}C]PtdCho and [^{14}C]PtdEtn by GF 109203X in NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were labeled with [^{14}C]choline (A) or [^{14}C]ethanolamine (B) for 60 h, followed by pretreatments of suspended fibroblasts with varying concentrations (0.1–75 μM) of GF 109203X for 15 min. This was followed by treatments with GF 109203X for another 30 min in the absence (●) or presence of 100 nM PMA (▲). Each point represents the mean ± S.E.M. of two experiments each performed in quadruplicate.

involved both phosphorylating and non-phosphorylating mechanisms. We should note here that after 60 min treatment, 25 μM GF 109203X caused about 80% inhibition of PMA-induced PtdCho hydrolysis, indicating that upon prolonged incubation the phosphorylating mechanism becomes the predominant regulatory mechanism (data not shown).

If C$_2$-ceramide specifically inhibits the phosphorylating mechanism, as the above results suggested, then one would expect no further modification of the maximal inhibitory effect of GF 109203X by ceramide. Indeed, in a similar experiment which is shown in Fig. 3A, 60 μM C$_2$-ceramide failed to modify the inhibitory effect of 25 μM GF 109203X on PMA-induced PtdCho hydrolysis (data not shown).

In summary, in NIH 3T3 fibroblasts C$_2$-ceramide was found to preferentially inhibit PMA-induced PtdEtn hydrolysis. In addition, GF 109203X also preferentially inhibited PMA-induced PtdEtn hydrolysis when the incubation time was relatively short. In taking into account relevant data in the literature, the best explanation we can presently offer for these phenomena is that C$_2$-ceramide inhibits PLD activity by preventing a PMA-induced, and possibly PKC-α-mediated, regulatory phosphorylation reaction. Experiments are underway in our laboratory to prove this possibility.

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