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The protein phosphatase inhibitor, okadaic acid, potentiates the stimulatory effect of phorbol ester on phosphatidylcholine synthesis, but not on phospholipid hydrolysis, in fibroblasts

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The potent protein phosphatase inhibitor, okadaic acid, was used to determine the possible role of protein phosphorylation reaction(s) in phorbol ester-induced synthesis and hydrolysis of phosphatidylcholine (PtdCho) in NIH 3T3 fibroblasts. Okadaic acid (2 μM) was found to enhance the stimulatory effects of lower concentrations (2.5–25 nM) of phorbol 12-myristate 13-acetate (PMA) on PtdCho synthesis, but not on PtdCho hydrolysis, after treatments for 30–60 min. These data support a view that in fibroblasts PMA stimulates only PtdCho synthesis, and not PtdCho hydrolysis, by a protein phosphorylation-dependent mechanism.

Okadaic acid; Phorbol ester; Phosphatidylcholine synthesis

1. INTRODUCTION

In most mammalian cell types examined, the potent tumor promoter, phorbol 12-myristate 13-acetate (PMA), stimulates the synthesis of CDP-choline, the rate-limiting substrate for phosphatidylcholine (PtdCho) synthesis (reviewed in [1–3]). Since the major cellular target of PMA is protein kinase C (PLC) [4–6], one would expect that regulation of PtdCho synthesis by the PMA/PKC system involves a protein phosphorylation reaction. However, attempts to show phosphorylation of choline phosphate cytidylyltransferase (EC 2.7.7.15) by PKC have failed so far [7–9]. In addition, recently Hatch et al. [10] showed that in hepatocytes, the protein phosphatase inhibitor, okadaic acid, inhibited PtdCho synthesis, implying that phosphorylation of a regulatory protein may actually inhibit PtdCho synthesis.

PMA, in addition to stimulating PtdCho synthesis, also stimulates PtdCho hydrolysis in virtually all cell types [3,11,12]. Furthermore, 1,2-diacylglycerol (1,2-DAG), often a degradation product of PtdCho hydrolysis, also stimulates PtdCho synthesis by a PKC-independent mechanism [13–16]. Thus, it is possible that 1,2-DAG mediates the stimulatory effect of PMA on PtdCho synthesis. Indeed, recently Utal et al. [9] suggested that in HeLa cells, 1,2-DAG has such mediatory role. On the other hand, we have presented evidence against a mediatory role of 1,2-DAG in NIH 3T3 fibroblasts [17,18].

Previous results obtained with membranes isolated from HL-60 cells [19] or lung fibroblasts [20] suggested that regulation of phospholipase D (PLD) activity by PMA/PKC did not involve a protein phosphorylation reaction. In an effort to understand the mechanism by which PMA regulates PtdCho synthesis in fibroblasts, here I examined the possible involvement of a protein phosphorylation mechanism. Using okadaic acid, a potent inhibitor of major phosphatases in fibroblasts [21], now I present evidence that in NIH 3T3 fibroblasts, regulation of PtdCho synthesis by PMA involves a protein phosphorylation reaction.

Abbreviations: PtdCho, phosphatidylcholine; PMA, phorbol 12-myristate 13-acetate; PLC, phospholipase D; PKC, protein kinase c; 1,2-DAG, 1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium.

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2. MATERIALS AND METHODS

2.1. *Materials*

PMA, PtdCho and Dowex-50W(H⁺ form) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); okadaic acid and tissue culture reagents were from Gibco (Grand Island, NY, USA); for comparative purposes, okadaic acid was also purchased from Kamiya Biomedical Co. (Thousand Oaks, CA, USA); [methyl-^{14}C]choline chloride (50 mCi/mmol) was from Amersham Corporation (Arlington Heights, IL, USA).

2.2. *Cell culture*

NIH 3T3 C-7 fibroblasts were continuously cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, penicillin (50 U/ml)/streptomycin (50 μg/ml), and glutamine (2 mM). Fibroblasts were seeded in 150-mm diameter plastic dishes, and growing (70–80%) cell populations were harvested (3–5 dishes) after two days in culture.

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2.3. *Measurement of PtdCho synthesis in intact fibroblasts*

For the measurement of PtdCho formation, fibroblasts were harvested by gentle scraping from the dish, followed by incubation of suspended fibroblasts (0.8–1.1 × 10⁶/ml; final volume 0.25 ml) with [methyl-^{14}C]choline (1.25–2 μCi/ml) in an incubator at 37°C for 15–60 min in the presence of PMA and/or okadaic acid, as indicated. Incubations were terminated by addition of 4 ml of chloroform/methanol (1/1, v/v). PtdCho was separated from other lipids on silica gel H plates by one-dimensional TLC using chloroform/methanol/28% ammonia (65/25/5, v/v/v).

2.4. *Measurement of PtdCho hydrolysis in intact fibroblasts*

NIH 3T3 fibroblasts were labelled with [methyl-^{14}C]choline for 48 h, washed, incubated for 3 h in fresh DMEM, and then harvested as above. Portions of suspended cells (0.9–1.2 × 10⁶/ml) were incubated (final volume 0.25 ml) in an incubator at 37°C in the presence of 20 mM unlabelled choline (to prevent phosphorylation of newly formed [^{14}C]choline) along with other agents, as specified. [^{14}C]Choline was separated from other degradation products by using Dowex-50W(H⁺) packed columns (Bio-Rad econo-columns; 1 ml bed volume) as described by Cook and Wakelam [22]. [^{14}C]Choline phosphate and [^{14}C]choline were eluted successively by 20 ml of water and 15 ml of 1 M HCl, respectively, and their [^{14}C] content was determined, as described earlier [17].

3. RESULTS AND DISCUSSION

Previous studies in our laboratory using NIH 3T3 fibroblasts [17] established that PMA has similar effects on the incorporation of [^{14}C]choline and $^{32}P_i$ into cellular PtdCho, and that PMA has no effect on the cellular uptake of [^{14}C]choline. For convenience, in the present study, [^{14}C]choline was used as the labelling agent to determine the extent of PtdCho synthesis. The concentration-dependent effect of PMA on PtdCho synthesis in the absence or presence of okadaic acid (2 μM) is shown in Fig. 1. PMA, at a concentration as low as 2.5 nM, stimulated PtdCho synthesis about 1.4-fold; a nearly maximal (3.25-fold) stimulation of PtdCho synthesis was observed with 50 nM TPA during a 60-min incubation period. In fibroblasts, in contrast to hepatocytes [10], okadaic acid (2 μM) only slightly (~20% or less) inhibited PtdCho synthesis in the absence of PMA. More importantly, okadaic acid enhanced the stimulatory effects of lower concentrations of PMA. Thus, at 2.5, 5.0, 10 and 25 nM concentrations of PMA, okadaic acid has 1.8-, 1.5-, 1.25- and 1.15-fold potentiating effects, respectively.

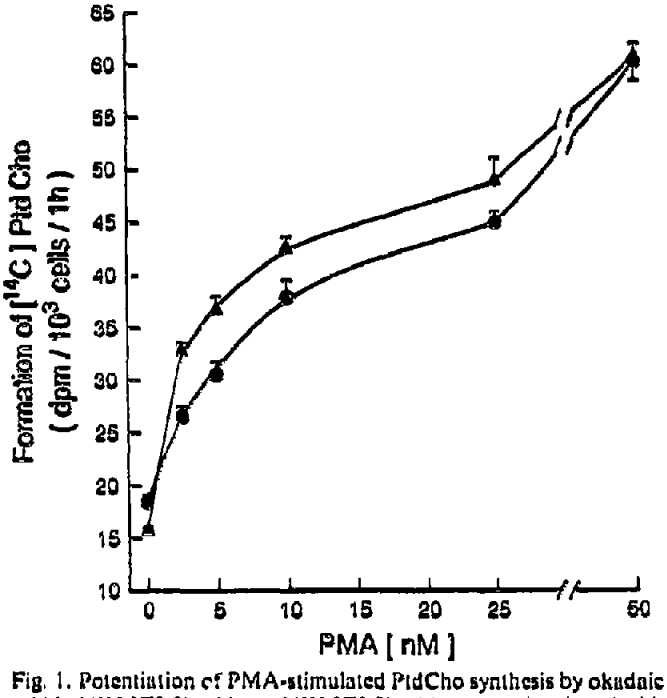

Fig. 1. Potentiation of PMA-stimulated PtdCho synthesis by okadaic acid in NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were incubated with [^{14}C]choline for 60 min in the presence of various concentrations of PMA without (●) or with (▲) 2 μM okadaic acid as described in section 2. Each point represents the mean ± S.E.M. of four incubations. Similar results were obtained in three other experiments.

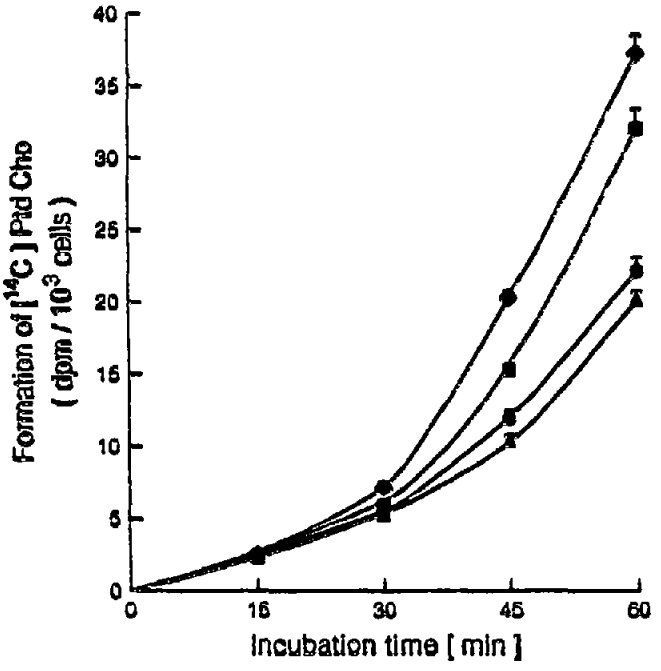

Fig. 2. Time dependence of the combined stimulatory effects of PMA and okadaic acid on PtdCho synthesis. NIH 3T3 fibroblasts were incubated with [^{14}C]choline for 15–60 min in the absence (●) or presence of 2 μM okadaic acid (▲), 2.5 nM PMA (■), or okadaic acid plus PMA (◆), as described in section 2. Each point represents the mean ± S.E.M. of four incubations. Similar results were obtained in two other experiments.

In the absence of okadaic acid, significant stimulation of PtdCho synthesis by 2.5 nM PMA could be observed only after a 45-min treatment period (Fig. 2). In the presence of okadaic acid, however, PMA had a detectable (~1.3-fold) stimulatory effect after 30 min of treatment (Fig. 2).

The possible potentiating effect of okadaic acid on PMA-stimulated PtdCho hydrolysis was examined in [^{14}C]choline-labelled cells. Previous studies from our laboratory [19] showed that in [^{14}C]choline-prelabelled NIH 3T3 cells, PMA-induced formation of [^{14}C]choline was due to PLD-mediated hydrolysis of the labelled PtdCho pool. In five experiments, we consistently ob-

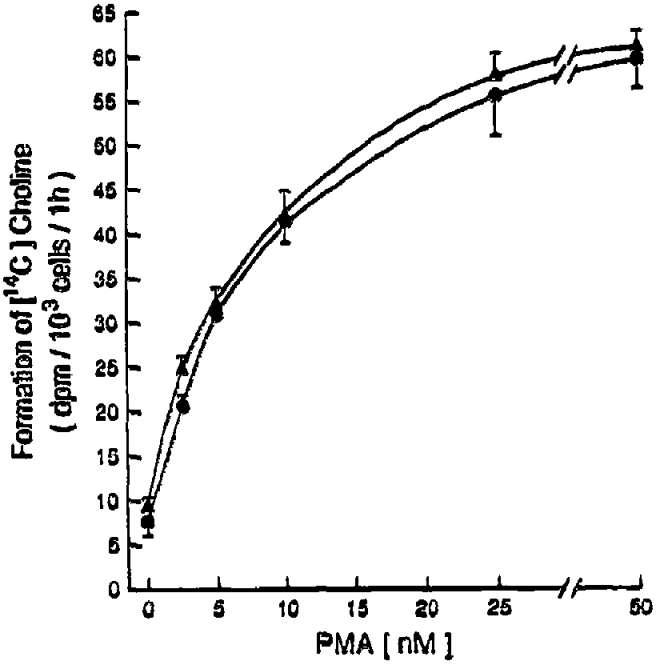

Fig. 3. Effects of okadaic acid and PMA on PtdCho hydrolysis. NIH 3T3 fibroblasts were labelled with [^{14}C]choline for 48 h, and then suspended cells were treated with 0–50 nM concentrations of PMA in the absence (●) or presence (▲) of 2 μM okadaic acid. The ^{14}C content of PtdCho was 1.16×10^6 dpm/10^6 cells. Each point represents the mean ± S.E.M. of four incubations. Similar results were obtained in four other experiments.

served 10–15% stimulation of [^{14}C]choline formation by 2 μM of okadaic acid in the absence of PMA (a representative experiment is shown in Fig. 3). While 2.5–50 nM concentrations of PMA significantly enhanced the hydrolysis of PtdCho, at none of these concentrations was the effect of PMA enhanced by okadaic acid above the small effect observed in the absence of PMA (Fig. 3). In addition, even the small stimulatory effects of okadaic acid, observed in the absence or presence of PMA after incubations for 60 min, became undetectable at shorter (45 min or less) incubation times (data not shown). These data support those earlier [19,20] that PMA-induced PtdCho hydrolysis probably does not involve protein phosphorylation. In addition, comparison of data in Figs. 1–3 clearly indicates that regulation of the synthesis and hydrolysis of PtdCho by PMA involves different mechanisms.

In fibroblasts, PMA also stimulates PLC-mediated hydrolysis of phosphatidylethanolamine, another potential source or 1,2-DAG [19,23]. However, at 15, 30, 45 and 60 min incubation times, 2 μM okadaic acid failed to enhance the stimulatory effects of 2.5–50 nM concentrations of PMA on the hydrolysis of this phospholipid (data not shown).

In conclusion, the ability of the specific protein phosphatase inhibitor, okadaic acid, to enhance the stimulatory effects of sub-optimal concentrations of PMA on PtdCho synthesis indicates that in fibroblasts the stimulation of PtdCho synthesis by PMA involves a protein phosphorylation reaction. At higher (50–100 nM) concentrations of PMA, PKC is apparently sufficiently activated to keep the (unknown) regulatory protein in the phosphorylated (and activated) state; this explains the inability of okadaic acid to enhance the effects of higher concentrations of PMA.

The inability of okadaic acid to potentiate the stimulatory effect of PMA on PtdCho hydrolysis confirms recent suggestions [19,20] that regulation of PLC activity by PKC may not involve a protein phosphorylation reaction. In addition, these data are consistent with our findings [17,18] that in fibroblasts, PMA-induced PtdCho synthesis is not causally related to increased phospholipid degradation.

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