Wortmannin has opposite effects on phorbol ester-induced DNA synthesis and phosphatidylcholine hydrolysis

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Abstract The tumor promoter phorbol 12-myristate 13-acetate (PMA) and hormonal activators of protein kinase C (PKC) commonly stimulate phospholipase D (PLD)-mediated formation of phosphatidic acid from phosphatidylcholine (PtdCho) in fibroblasts and other cell types. On the basis that phosphatidic acid is a mitogen, PLD is often considered to have a major role in the regulation of cell growth by PKC activators. However, we found that in NIH 3T3 fibroblasts wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K), strongly inhibited DNA synthesis induced by 100 nM PMA, while it actually enhanced PMA-stimulated PtdCho hydrolysis. These results indicate that stimulation of PLD activity is either not required or not sufficient for the mitogenic action of PMA.

Key words: Wortmannin; Phorbol ester; DNA synthesis

1. Introduction

Phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine (PtdCho) produces phosphatidic acid (PtdOH) [1,2], a potent mitogen in several cell types, including fibroblasts [2–8]. Furthermore, PtdOH can be degraded to 1,2-diacylglycerol which activates protein kinase C (PKC), an important regulator of cell growth [9]. For these reasons, PLD could play some role in the regulation of cell growth by the PKC system [10]. However, this question is difficult to study because the mechanism of mitogenic action of PMA is unknown, and no specific inhibitor of PLD is presently available.

In NIH 3T3 fibroblasts, the PKC system is clearly a major regulator of PLD. In this system, both the tumor promoter phorbol 12-myristate 13-acetate (PMA) and hormonal activators of PKC can greatly enhance PLD activity for which PtdCho serves as a major, although not the only, substrate [11,12]. Earlier, we demonstrated that the stimulatory action of PMA on phospholipid hydrolysis in these cells is accompanied by significant elevation of cellular levels of PtdOH [13]. The initial aim of this work was to determine whether PtdOH is a mediator of the mitogenic effect of PMA. For this purpose, we first searched for an inhibitor of PMA-induced DNA synthesis. Surprisingly, during this search we found, as reported here, that the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin [14,15] strongly inhibited PMA-induced DNA synthesis, but it had no such inhibitory effect on PMA-stimulated PtdCho hydrolysis.

2. Experimental

2.1. Materials

PMA, wortmannin and Dowex-50W (H^+ form) were purchased from Sigma; [methyl-^{14}C]choline chloride (50 mCi/mmol), [1-^{14}C]palmitic acid (60 mCi/mmol) and [methyl-^{3}H]thymidine (85 mCi/mmol) were bought from Amersham; phosphatidylethanol (PtdEtOH) was from Avanti Polar Lipids, Inc; and tissue culture reagents were purchased from GIBCO BRL.

2.2. Cell culture

NIH 3T3 clone-7 fibroblasts, kindly provided by Dr. Douglas R. Lowy (National Cancer Institute, NIH, Bethesda, MD), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin-streptomycin (50 U/ml and 50 μg/ml, respectively) and glutamine (2 mM).

2.3. Labeling of cellular DNA with [^{3}H]thymidine

NIH 3T3 fibroblasts were grown in 12-well tissue culture dishes to ~30–40% confluency in the presence of 10% serum, followed by incubation of fibroblasts in serum-free medium for 24 h. Subsequently, fibroblasts were treated (in serum-free medium) first with wortmannin for 20 min, and then with PMA and/or wortmannin for 16 h. Finally, incubations were continued in the presence of [methyl-^{3}H]thymidine (1 μCi/ well) for 60 min. Fibroblasts were washed twice with phosphate-buffered saline and then four times with 5% trichloroacetic acid. The acid- insoluble material was redissolved in 0.3 M sodium hydroxide, and an aliquot was taken to measure DNA-associated ^{3}H activity in a liquid scintillation counter.

2.4. Measurement of PtdEtOH formation in NIH 3T3 fibroblasts

Fibroblasts were grown in 12-well tissue culture dishes for 48 h to ~80% confluency and labeled with [1-^{14}C]palmitic acid (0.5 μCi/ml) for the last 24 h. Fibroblasts were washed, incubated in fresh medium for an additional 2-h period (to lower the amount of unesterified labeled palmitic acid) and then treated first with 3 μM wortmannin for 20 min, and then with 100 nM PMA and/or wortmannin for an additional 20-min period in the presence of 200 mM ethanol. Incubations were terminated by scraping the cells 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) with a solvent system of chloroform/methanol/acetone/acetic acid/water (50:10:15:10:2, v/v/v/v/v).

2.5. Determination of PtdCho hydrolysis in NIH 3T3 fibroblasts

Fibroblasts were seeded in 150 mm-diameter plastic dishes and were incubated with [methyl-^{14}C]choline (0.35 μCi/ml) for 48 h. Cells were washed and then incubated in the corresponding fresh medium for 3 h; this step reduces the cellular level of [^{14}C]choline, also a product of PLD action, by a factor of 3–4-fold. Cells were harvested by gentle scraping from 3–6 dishes and washed; this procedure further reduces the background level of [^{14}C]choline, but washed cells retain sensitivity to PMA. Suspended cells were first treated with wortmannin for 20 min, then incubations were continued for 30 min in the absence or presence of 10–100 nM PMA and/or wortmannin. Separation of [^{14}C]choline

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PLD, phospholipase D; PI3K, phosphatidylinositol 3-kinase; PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid.

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from other products was performed on Dowex-50W(H^+)-packed columns as previously described [16].

3. Results and discussion

Treatment of NIH 3T3 fibroblasts with 100 nM PMA for 16 h in serum-free medium resulted in ~23-fold increase in DNA synthesis (Fig. 1). Fifty nM and 3 μM concentrations of wortmannin inhibited PMA-induced DNA synthesis by ~75 and 86%, respectively (Fig. 1). It should be emphasized that maximal inhibitory effects of wortmannin required addition of the drug ~10–20 min prior to PMA. When wortmannin and PMA were added to the wells simultaneously, even 3 μM wortmannin failed to exert any effect on PMA-stimulated incorporation of [^{3}H]thymidine into DNA (data not shown).

Treatment of serum-starved fibroblasts with 10% serum for 16 h enhanced DNA synthesis ~85-fold; wortmannin at both 50 nM and 3 μM concentrations failed to significantly decrease serum-induced DNA synthesis (data not shown). These observations indicated that inhibition of PMA-induced DNA synthesis was not due to some non-specific toxicity of wortmannin. On the other hand, 50 nM and 3 μM concentrations of wortmannin inhibited the stimulatory effect (~35-fold) of platelet-derived growth factor (50 ng/ml) on DNA synthesis by 73 and 88%, respectively (data not shown). Thus, wortmannin exhibits very similar inhibitory effects on PMA- and platelet-derived growth factor-induced DNA synthesis.

Synthesis of phosphatidylalcohol from phospholipids and alcohol is a specific function of activated PLD [1,2]. Presently, addition of 100 nM PMA to [^{14}C]palmitic acid-labeled NIH 3T3 fibroblasts enhanced the synthesis of phosphatidylethanol (PtdEtOH) ~3.6-fold (Fig. 2). Even a high concentration (3 μM) of wortmannin failed to inhibit, in fact it appeared to slightly enhance, PMA-induced formation of PtdEtOH (Fig. 2).

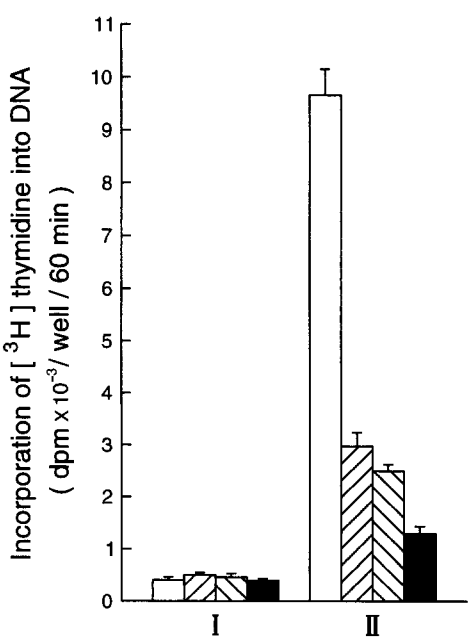

Fig. 1. Inhibition of PMA-induced DNA synthesis by wortmannin in NIH 3T3 fibroblasts. Serum-starved fibroblasts were incubated for 16 h in the absence (I) or presence (II) of 100 nM PMA; there was either no further addition (□), or fibroblasts were additionally treated with 0.05 μM (▨), 0.2 μM (▩) or 3 μM (■) concentrations of wortmannin; when present, wortmannin was added 20 min prior to PMA. Data represent the mean ± S.E. of three experiments performed on the same day. Similar results were obtained in three other experiments, each performed in triplicate.

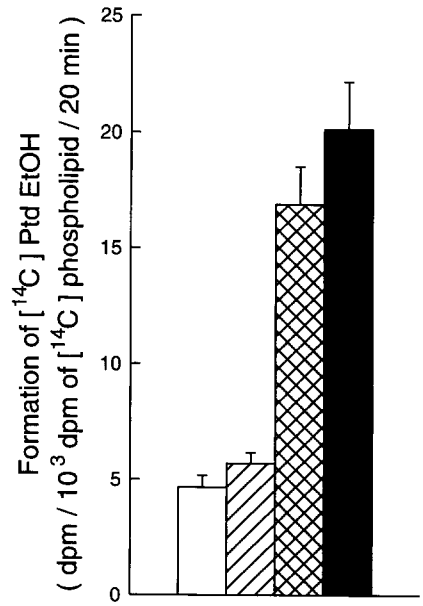

Fig. 2. Combined effects of PMA and wortmannin on PtdEtOH formation. Attached NIH 3T3 fibroblasts were prelabeled with [^{14}C]palmitic acid, and then incubated for 20 min in the absence (□) or presence of 3 μM wortmannin (▨), 100 nM PMA (▩), or wortmannin + PMA (■). When present, wortmannin was added 20 min prior to PMA. Data represent the mean ± S.E. of four incubations performed on the same day with the same passage of cells. Similar results were obtained in another experiment performed in triplicate.

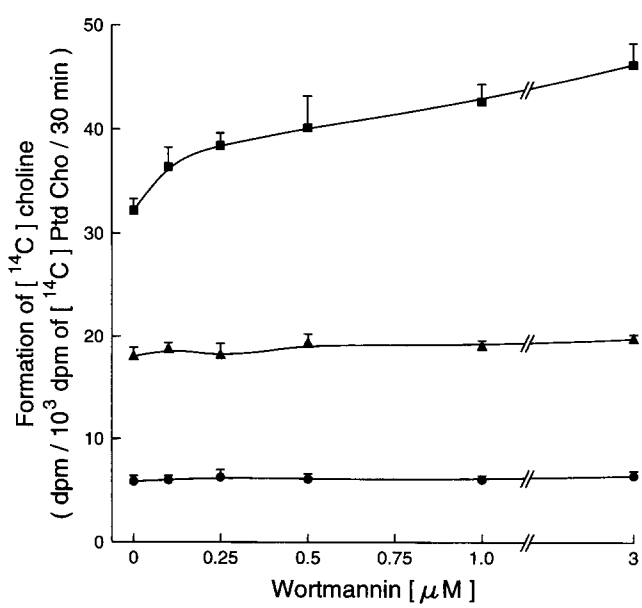

Fig. 3. Concentration-dependent effect of wortmannin on PtdCho hydrolysis. NIH 3T3 fibroblasts were labeled with [^{14}C]choline for 48 h, followed by pretreatment of suspended cells for 20 min with 0.1–3 μM concentrations of wortmannin. Then fibroblasts were incubated (without removing wortmannin) for a 30-min period in the absence (●) or presence of 10 nM PMA (▲) or 100 nM PMA (■). Each point represents the mean ± S.E. of four independent incubations. Similar results were obtained in two other experiments, each performed in triplicate.

In [^{14}C]choline-labeled NIH 3T3 fibroblasts, PMA-induced loss of [^{14}C]PtdCho occurs by a PLD-mediated hydrolytic process [10]. In these fibroblasts, 0.1–3.0 μM concentrations of wortmannin again tended to enhance, rather than decrease, PMA-induced hydrolysis of [^{14}C]PtdCho when PMA was added at a maximally effective concentration (100 nM) (Fig. 3). We should add here that this large stimulatory effect of 100 nM PMA was sustained for ~3 h, while the slight potentiating effect of wortmannin became progressively less pronounced after 30 min incubation. However, even after a treatment for 3 h, wortmannin still had a potentiating, rather than an inhibitory, effect on PMA (100 nM)-induced PtdCho hydrolysis (data not shown). At a 10 nM concentration of PMA, wortmannin had no potentiating effect on PtdCho hydrolysis (Fig. 3).

It is possible that chronic (16 h) treatment of fibroblasts with PMA in the absence of serum results in the activation of PLD by a different mechanism which might be inhibited by wortmannin. We examined this possibility in the following way. NIH 3T3 fibroblasts were labeled with [^{14}C]Cho for 60 h and treated with 100 nM PMA in the absence or presence of 3 μM wortmannin (wortmannin being added to the cells 20 min prior to PMA) for the last 16 h of the labeling period. To facilitate comparison of this experiment with that described in Fig. 1, treatments of attached fibroblasts with PMA were performed in serum-free medium. When the washed untreated and PMA-treated attached fibroblasts were compared, the latter exhibited ~1.5–1.6-fold increase in [^{14}C]PtdCho hydrolysis; the co-presence of wortmannin during the entire treatment period failed to prevent this small stimulatory effect of PMA (data not shown).

In summary, we showed here that in NIH 3T3 fibroblasts wortmannin inhibits PMA-induced DNA synthesis but not PMA-stimulated PtdCho hydrolysis. Accordingly, in these cells activation of PLD is either not required or not sufficient for the stimulatory effect of PMA on DNA synthesis. The results also suggest that PI3K may be required for the mitogenic action of PMA; however, this possibility remains to be proven.

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