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Comparison of phospholipase D activity in vasopressin- and phorbol ester-stimulated fibroblasts

Chunfa Huang[a], Robert L. Wykle[a] and Myles C. Cabot[b]

[a]*Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Medical Center Boulevard, Winston-Salem, NC 27157-1016, USA* and [b]*John Wayne Cancer Institute, 2200 Santa Monica Boulevard, Santa Monica, CA 90404, USA*

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Phospholipase D (PLD) activation by vasopressin (VP) was compared to activation by TPA in REF52 cells prelabeled with [^{3}H]glycerol and [^{14}C]myristic acid. Upon VP-treatment, the formation of [^{3}H] and [^{14}C]phosphatidic acid (PA) and phosphatidylethanol (PEt) was accompanied by the loss of radioactivity from PC and PI. However, upon TPA-treatment, radioactivity was lost from PC only. No significant changes of phosphatidylethanolamine and phosphatidylserine were detected in the same samples. The inclusion of 5 μM staurosporine for 10 min diminished the production of [^{3}H]PEt and [^{14}C]PEt by 27% and 53% in VP-treated cells, and by 100% and 75% in TPA-treated cells, respectively. Adding 1 mM EGTA to chelate extracellular Ca^{2+} inhibited [^{3}H]PEt by approximately 31% and [^{14}C]PEt by 17% after VP-stimulation. In contrast, EGTA had no effect on TPA-stimulation. The data suggest that REF52 cells contain dual PLD activities. The first is stimulated only by VP, requires Ca^{2+} and hydrolyzes PI. The second is stimulated by both TPA and VP, activated by protein kinase C and hydrolyzes PC.

Phospholipase D; Vasopressin; Phorbol ester; Fibroblast

1. INTRODUCTION

Many agonists (hormones, neurotransmitters, growth factors, tumor promotors, etc.) are thought to elicit cell physiological responses through the action of phospholipase A_2, C or D, which cleaves cellular phospholipids and generates second messenger molecules [1–5]. Two major classes of signal transduction enzymes, phospholipase A_2 and C, have received much more attention. Phospholipase A_2 forms lysophospholipids and releases arachidonic acid leading to the production of platelet activated factor, prostaglandins, leukotrienes and other eicosanoides that are involved in a variety of pathophysiological events [5]. Phospholipase C hydrolyzes phosphatidylinositol-4,5-bisphosphate to produce inositol-1,4,5-trisphosphate and diacylglycerol (DG), which mobilize intracellular calcium and activate protein kinase C [1,3]. Phospholipase D (PLD) catalyzes the hydrolysis of phospholipids to form PA for which a role in signal transduction has not been conclusively established. However, several investigators have reported dephosphorylation of PA by PA phosphatase to yield DG [6–8] and the exposure of cells to PA can elicit several biological responses [9] which suggest a possible second messenger role in cellular responses.

In the previous studies, we have demonstrated the degradation of PC in REF52 cells stimulated by VP or TPA [10–12], but the metabolic pathway was not fully elucidated. The purpose of the present studies was to compare PLD activation by either hormone (VP) or tumor promotor (TPA) and to assess the role of Ca^{2+} and protein kinase C in response to the two dissimilar stimuli.

Correspondence address: C. Huang, Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Medical Center Boulevard, Winston-Salem, NC 27157-1016, USA. Fax: (1) (919) 716-7671.

Abbreviations: REF52, rat embryo fibroblasts; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VP, vasopressin; PEt, phosphatidylethanol; PA, phosphatidic acid; PLD, phospholipase D; PC, phosphatidylcholine; PI, phosphatidylinositol; DG, diacylglycerol. In naming of PC, PI, PA, PEt and DG, we do not distinguish between the type of aliphatic linkage to glycerol (ester, ether).

2. MATERIALS AND METHODS

2.1. *Materials*

[2-^{3}H]Glycerol (15 Ci/mmol) was a product of American Radiolabeled Chemicals (St. Louis, MO); [1-^{14}C]myristic acid (58 mCi/mmol) was purchased from Du Pont/New England Nuclear. Powdered culture media were obtained from Gibco (Gaithersburg, MD). Bovine serum albumin and [Arg^8]VP (acetate salt) were purchased from Sigma. TPA was a product of Chemicals for Cancer Research (Eden Prairie, MN). Fetal bovine serum was supplied by HyClone (Logan, UT). Commercial phospholipids were from Avanti Polar Lipids, and neutral lipids were from Nu Chek Prep. Silica gel thin layer chromatography plates were a product of Analtech. Solvents were purchased from Fisher Scientific. Staurosporine was kindly provided by Dr. Hiroshi Kase (Kyowa Kogyo Co., Ltd., Tokyo Research Laboratories, Tokyo).

2.2. *Culture, radiolabeling, and treatments of REF52 cells*

REF52 cells were obtained and cultured as previously described [11]. Stock cultures were maintained in 75-mm flasks and subcultured into 6-well plates or 35-mm dishes for experiments. REF52 cells were

Table I

The formation of DG and PA and the degradation of phospholipids in REF52 cells by VP- or TPA-stimulation

Lipid	^{3}H (dpm/dish)			^{14}C (dpm/dish)		
	Control	VP	TPA	Control	VP	TPA
DG	1,003 ± 37	2,077 ± 87	1,222 ± 123	3,274 ± 152	11,750 ± 86	8,120 ± 90
PA	1,851 ± 202	5,472 ± 316	3,895 ± 165	4,037 ± 163	11,234 ± 327	11,118 ± 789
PC	58,086 ± 3,375	58,569 ± 6,008	58,986 ± 1,535	408,024 ± 5,190	385,380 ± 26,049	373,865 ± 3,672
PE*	40,582 ± 1,375	39,489 ± 1,909	41,603 ± 1,587	35,489 ± 1,144	32,331 ± 1,331	38,758 ± 2,850
PI + PS	30,249 ± 2,804	25,891 ± 1,540	33,100 ± 1,222	15,702 ± 1,655	13,106 ± 1,421	14,808 ± 1,016
PI	17,838 ± 977	11,359 ± 758	17,238 ± 1,616	14,780 ± 1,402	14,718 ± 1,394	13,764 ± 1,879

REF52 cells were prelabeled with [^{3}H]glycerol (4.9 μCi/ml) and [^{14}C]myristic acid (0.5 μCi/ml) and treated with 100 ng/ml VP or 80 nM TPA for 10 min. Cellular lipids were extracted, resolved and quantitated as described in section 2. Data represent the mean of four determinations. *PE, phosphatidylethanolamine; PS, phosphatidylserine.

used at passage 8–15. Cultures were incubated with [^{3}H]glycerol and [^{14}C]myristic acid for 24 h in 5% fetal bovine serum medium beginning one day before confluency. After removal of the radiolabeling medium, cells were rinsed twice with serum- and isotope-free medium containing 1 mg bovine serum albumin/ml, and equilibrated in the same medium for another 2 h. For the experiments, the cells were treated as described in the figure legends and tables. Total cellular lipids were extracted by the Bligh and Dyer procedure [13], modified to contain 1% HCl in methanol.

2.3. *Cellular lipid analysis*

Radiolabeled PA, DG, PEt, PC, PI, PI plus phosphatidylserine, and phosphatidylethanolamine were resolved by thin layer chromatography as previously described [8,11]. The radioactivity was quantitated by liquid scintillation spectrometry.

3. RESULTS

3.1. *The degradation of phospholipid by either VP- or TPA-stimulation*

The experiments presented here were designed to compare PLD activity induced by agonists of dissimilar nature: VP and TPA. We employed [^{3}H]glycerol and [^{14}C]myristic acid to selectively label the different phospholipids in REF52 cells. The data in Table I show that either VP or TPA induced a marked formation of radiolabeled PA and DG. VP treatment elicited 3.0-fold increases in [^{3}H]PA and [^{14}C]PA and concomitant increases in [^{3}H]DG (2.1-fold) and [^{14}C]DG (3.6-fold), respectively. The incubation of REF52 cells with TPA resulted in increases in [^{3}H]PA (2.1-fold) and [^{14}C]PA (2.8-fold) and accompanying increases in [^{3}H]DG (1.2-fold) and [^{14}C]DG (2.5-fold). One of the most important differences was that the formation of radiolabeled PA and DG was paralleled by the degradation of radiolabeled PC and PI in VP-induced cells, but by the degradation of radiolabeled PC only in TPA-induced cells. No significant changes were observed in the breakdown of phosphatidylethanolamine and phosphatidylserine of the same samples (Table I).

3.2. *The effect of staurosporine on VP- or TPA-induced PLD activity*

We further detected the activity of PLD by determining the formation of PEt in double labeled REF52 cells stimulated by VP or TPA in the presence of 2% ethanol.

Table II

The hydrolysis of PC and PI in REF52 cells by VP- or TPA-stimulation in the presence or absence of staurosporine

Cellular treatment	PC (dpm/well)		PI (dpm/well)	
	^{3}H	^{14}C	^{3}H	^{14}C
Control	57,744 ± 3,050	408,017 ± 12,956	17,733 ± 823	14,989 ± 195
E	57,407 ± 4,159	407,739 ± 9,412	18,292 ± 524	15,124 ± 325
VP + E	55,323 ± 2,190	390,558 ± 7,530	14,514 ± 673	10,052 ± 849
VP + E + St	58,306 ± 1,821	410,901 ± 6,680	16,761 ± 733	12,506 ± 837
TPA + E	55,885 ± 2,511	397,985 ± 3,862	17,721 ± 521	14,165 ± 650
TPA + E + St	57,786 ± 3,065	410,487 ± 4,358	17,771 ± 844	14,926 ± 691

REF52 cells were double-labeled by incubation with [^{3}H]glycerol (6.6 μCi/ml) and [^{14}C]myristic acid (0.5 μCi/ml). Cells were treated with 100 ng/ml VP or 80 nM TPA medium containing 2% ethanol (E) in the presence or absence of 5 μM staurosporine (St) for 10 min. PC and PI were resolved and quantitated as described in section 2. The data are the average values from four determinations.

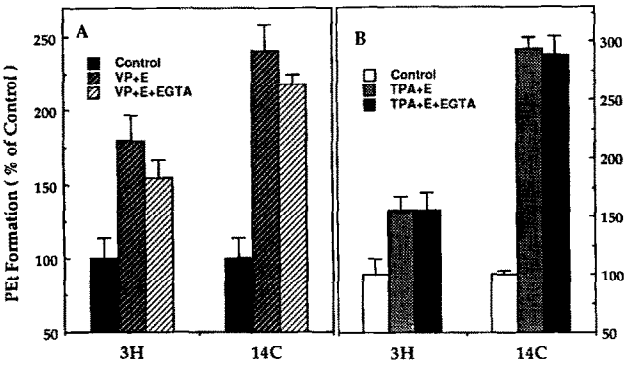

Fig. 1. The formation of [^{3}H]PEt and [^{14}C]PEt in REF52 cells by VP- or TPA-induced in the presence or absence of staurosporine. REF52 cells were double-labeled by incubation with [^{3}H]glycerol (6.6 μCi/ml) and [^{14}C]myristic acid (0.5 μCi/ml). Cells were treated with 100 ng/ml VP or 80 nM TPA medium containing 2% ethanol (E) in the presence or absence of 5 μM staurosporine (St) for 10 min. Cellular lipids were extracted, and radiolabeled PEt was resolved and quantitated as described in section 2. The data are the average values from four determinations. Control: ^{3}H, 2,542 ± 314 dpm/dish, and ^{14}C, 3,652 ± 320 dpm/dish.

Upon 10 min stimulation, VP initiated 2.3- and 3.0-fold increases in [^{3}H]PEt and [^{14}C]PEt, and TPA caused 1.4- and 2.3-fold increases in [^{3}H]PEt and [^{14}C]PEt respectively (Fig. 1). Measuring the concomitant conversion of PC and PI in the transphosphatidylation reaction showed that PC and PI were decreased upon VP-stimulation, but only PC upon TPA-stimulation (Table II). When 5 μM staurosporine was added to the agonist-containing medium for 10 min, PLD activity was inhibited differentially depending upon the agonist used. The weak response to VP-treatment with staurosporine diminished the formation of [^{3}H]PEt and [^{14}C]PEt by 27% and 53% (Fig. 1A), whereas in the strong response to TPA-treatment, it inhibited the formation of [^{3}H]PEt and [^{14}C]PEt by 100% and 75% (Fig. 1B). These data suggest that PLD activity can be regulated by a protein kinase C-dependent pathway in either VP- or TPA-stimulated REF52 cells, however, activation of PLD by VP is, in part, brought about by a protein kinase C-independent mechanism.

3.3. *The effect of Ca^{2+} on VP- or TPA-induced PLD activity*

In order to more clearly compare the metabolic pathways involved in the hydrolysis of phospholipids, experiments were designed to investigate the Ca^{2+}-requirement of PLD activity in VP- or TPA-induced REF52 cells. Incubations were performed in medium containing 2% ethanol and either VP or TPA in the presence or absence of 1 mM EGTA for 10 min. The inclusion of EGTA decreased the formation of [^{3}H]PEt and [^{14}C]PEt to 31% and 17% of the VP-induced response (Fig. 2A), respectively. In contrast, the formation of radiolabeled-PEt by TPA-stimulation was similar in the presence or absence of EGTA (Fig. 2B).

4. DISCUSSION

Several groups have demonstrated a PLD pathway for PC degradation in agonist-stimulated cells [6–8,14–17]. Studies of PC metabolism in intact cells were facilitated by employing radiolabeled myristic acid [10,14]. However, prelabeling with glycerol resulted in much higher incorporation of the radiolabeled precursor into the cellular PI fraction (Table I) [8,17]. Using this double-labeling approach helps to trace the metabolism of different phospholipids in the same system. The results reported herein indicate that PLD acts on PC in REF52 cells treated with VP and TPA. Meanwhile, we found that the accumulation of double-labeled PA or PEt by VP-activated PLD was partially associated with the hydrolysis of PI, in contrast, TPA completely failed to induce PI degradation (Tables I and II). On the other hand, we have recently observed that VP induces polyphosphoinositides and PC degradation in REF52 cells [10–12]. With regard to phorbol ester-induced turnover of phosphoinositides, several investigators have indicated these agents do not stimulate the hydrolysis of polyphosphoinositides [18,19]. Taken together, the results suggest that the difference of phospholipid degradation in response to VP and TPA may be relevant to extracellular signals of hormone and tumor promotors, and that the dual pathways elicited by VP imply a hormone self-adjusted function in lipid metabolism and cell responses.

The mechanism of PLD activation by agonists has been attributed to various mechanisms. PLD activation by TPA-stimulation is thought to be mediated by a protein kinase C-dependent pathway [3,6,10,14–16].

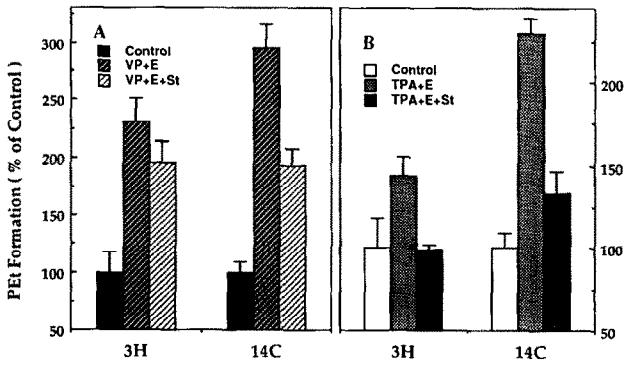

Fig. 2. The formation of [^{3}H]PEt and [^{14}C]PEt in REF52 cells by VP- or TPA-induced in the presence or absence of EGTA. REF52 cells were double-labeled by incubation with [^{3}H]glycerol (7.2 μCi/ml) and [^{14}C]myristic acid (0.8 μCi/ml) and treated with 100 ng/ml VP or 80 nM TPA medium containing 2% ethanol (E) in the presence or absence of 1 mM EGTA for 10 min. Cellular lipids were extracted, and radiolabeled PEt was resolved and quantitated as described in section 2. The results represent the mean of triplicate, and two experiments yielded similar results. Control: ^{3}H, 3,432 ± 484 dpm/dish, and ^{14}C, 5,253 ± 177 dpm/dish.

However, experiments have been carried out in hepatocytes indicating that VP-induced PC hydrolysis by PLD is G-protein coupled [20]. Our previous results indicate that VP-elicited PC degradation by PLD is in part regulated by protein kinase C [11], TPA-induced PEt formation is independent of extracellular calcium in several cell lines [8]. Using staurosporine and EGTA, the current report has demonstrated that VP induces the concurrent dual PLD activities that are protein kinase C- and Ca^{2+}-dependent. In contrast, TPA activates a protein kinase C-dependent PLD activity only, and not the Ca^{2+}-dependent activity (Figs. 1 and 2). Reinhold et al. [21] reported that there are three separable mechanisms to activate PLD in human neutrophils. The phenomenon of agonist-selective activation provides an interesting clue into the physiological role of PLD in signal transduction.

The above data implicating multiple PLD-mediated pathways were based on: (i) the concomitant degradation of various phospholipids in the formation of PA and PEt; (ii) the inhibition of PLD in response to some agonists by staurosporine; and (iii) the degree of Ca^{2+}-requirement in PLD action. Although the recent reports demonstrated a PI-PLD and a PC-PLD in Madin Darby canine kidney cells [18] and a PLD hydrolyzing phosphatidylethanolamine in NIH 3T3 fibroblasts [22], the dual PLD activities which require protein kinase C and Ca^{2+}, and hydrolyze PC and PI in REF52 cells provide a system to explore the complicated relationship between metabolic pathways and multiforms of PLD.

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