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The phosphatidylcholine pathway of diacylglycerol formation stimulated by phorbol diesters occurs via phospholipase D activation

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Agonist-induced degradation of phosphatidylcholine (PC) is of interest as this pathway of diacylglycerol (DG) generation may provide added opportunities for the regulation of protein kinase C (PKC). In REF52 cells [^{3}H]myristic acid is preferentially incorporated into PC; this, coupled with the use of [^{3}H]choline, allows for quantitation of both the water-soluble and the lipid products generated when PC is degraded. In cells prelabeled with [^{3}H]choline, TPA stimulated a time-dependent release, into the medium, of choline and not phosphocholine or glycerophosphocholine. Treatment of [^{3}H]myristic acid-labeled cells with either phorbol diesters, *sn*-1,2-dioctanoylglycerol, or vasopressin elicited the formation of labeled phosphatidate (PA) and DG. The temporal pattern of PC hydrolysis in cells treated with TPA is indicative of a precursor (PA)-product (DG) relationship for an enzymatic sequence initiated by phospholipase D. Adding propranolol, a phosphatidate phosphohydrolase inhibitor, eliminated TPA-induced DG formation, whereas PA generation was unaffected. From these data we conclude that TPA elicits DG formation from PC by the sequential actions of phospholipase D and phosphatidate phosphohydrolase.

Phosphatidylcholine; Diacylglycerol; Phospholipase D; Phorbol diester; Vasopressin; (REF52 cell)

1. INTRODUCTION

The action of many cellular agonists is transmitted via a receptor-mediated response that elicits phosphoinositide hydrolysis by a PIP_2-specific phospholipase C [1]. This reaction yields DG and inositol phosphates, which together activate PKC [2,3]. TPA, because of its metabolic stability and structural analogy to DG, circumvents the PIP_2 pathway of DG generation and interacts directly with PKC [4,5]. TPA has therefore been a valuable tool for investigating the cellular aspects of PKC activation. The classical scheme involving PIP_2 as the sole source of DG second messengers has recently been challenged. New research has revealed a cellular pathway for the agonist-induced generation of DG from PC [6–9]; however, the enzymatic avenue of DG formation has not been clarified.

In a previous study we have shown that serum, VP and TPA induce PC degradation and the collateral formation of DG in cultured REF52 cells [9]. Cell treatment was accompanied by the release of [^{3}H]choline to the culture medium [9], suggesting that phospholipase D was the initial enzyme involved in the evolution of DGs. The study of PC degradation in cultured cells has been hindered due to a lack of precursors that are

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Abbreviations: PIP_2, phosphatidylinositol 4,5-bisphosphate; DG, diacylglycerol; PKC, Ca^{2+}-activated, phospholipid-dependent protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PC, phosphatidylcholine; BSA, bovine serum albumin; VP, vasopressin; PA, phosphatidate; TLC, thin-layer chromatography. In the naming of PC, PA and DG we do not distinguish between the type of aliphatic linkage to glycerol (ester, ether)

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specific for labeling the aliphatic portion of the molecule. In this work we have employed [^{3}H]myristic acid, which is preferentially incorporated into PC of REF52 cells, to determine the enzymatic sequence of events responsible for phorbol diester-induced formation of DG. Our study indicates that DGs arise from PC via the initial action of phospholipase D.

2. MATERIALS AND METHODS

2.1. *Materials*

[9,10(*N*)-^{3}H]Myristic acid (41 Ci/mmol) was purchased from Amersham, and [*methyl*-^{3}H]choline chloride (80 Ci/mmol) was from New England Nuclear. TPA was purchased from Chemicals for Cancer Research (Eden Prairie, MN). Propranolol, cell culture media and VP were purchased from Sigma. Silica gel G TLC plates were from Analtech, Inc. (Newark, DE), and solvents were purchased from Burdick & Jackson and J.T. Baker. Fetal bovine serum was from HyClone (Logan, UT). Lipid standards for TLC were purchased from Avanti Polar Lipids (Birmingham, AL) and Nu Chek Prep (Elysian, MN).

2.2. *Cell culture, radiolabeling, and treatment*

The rat embryo-derived cell line, REF52, provided by Dr D.B. McClure (Division of Molecular and Cellular Biology, Eli Lilly and Company, Indianapolis, IN), was cultured as previously described [9–11]. Cells were used at passages 5 through to 15.

Confluent monolayers were rinsed twice in serum-free medium before addition of isotopes. Cells were labeled for 60 min with [^{3}H]myristic acid (0.5–1.0 μCi/ml serum-free medium containing 0.1 mg BSA per ml, designated medium A). After removal of isotope, monolayers were rinsed twice in serum-free medium containing 1.0 mg BSA/ml (medium B), and incubated in the same for 60 min. Following one rinse with medium B, TPA and VP were added in medium B. TPA (8×10^{-8} M) was added as previously described [9]; VP (100 ng/ml) was added in 0.05 N acetic acid. Cells were prelabeled with [^{3}H]choline (1.0 μCi/ml medium) for 48 h and then prepared for experiments as described above.

2.3. *Lipid analysis*

Total cellular lipids were extracted by a modified [9] Bligh and Dyer [12] procedure. For [^{3}H]myristic acid-labeled experiments, neutral lipids and phospholipids (with the exception of PA) were resolved by TLC using the solvent systems described previously [9]. PA was resolved in a solvent system containing chloroform/pyridine/70% formic acid (50:30:7, v/v). The individual lipids, cochromatographed with standards, were visualized in iodine vapor and radioactivity was determined by liquid scintillation spectrometry [13]. Plates run in the pyridine system were left under the fume hood overnight and lipids were visualized the next day. [^{3}H]Choline-labeled metabolites released into the medium were identified by TLC and quantitated as described [9].

3. RESULTS

Approx. 40% of the [^{3}H]myristic acid added to the culture medium was taken up by REF52 cells after 60 min. The distribution of radioactivity in cellular lipids is shown in table 1; PC accounted for 82% of the total. Phospholipid radioactivity accounted for approx. 90% of the total, and of this, the majority of [^{3}H]myristate (>90%) was localized in the PC fraction.

PC hydrolysis was studied by analysis of both hydrophilic and hydrophobic degradation products. In cells prelabeled with [^{3}H]choline, TPA treatment caused a time-dependent release of water-soluble [^{3}H]choline-containing metabolites into the medium, that by 60 min was approx. 4-fold higher than in control cells (fig.1, inset). Analysis of the radioactivity released showed that the increase elicited by TPA was due mainly to an increase in [^{3}H]choline (fig.1, left panel) and that although TPA caused an increase in phosphocholine release (middle panel), the contribution to the total was not significant (<4%). Tritium in glycerophosphocholine was not altered by TPA treatment. Therefore, the data in fig.1 remained unaltered. Therefore, the data in fig.1 reveal that ~96% of the TPA-induced release of tritium is in the form of [^{3}H]choline. As hydrolysis of the choline moiety should be accompanied by the formation of PA, cells prelabeled with [^{3}H]myristic

Table 1

Distribution of [^{3}H]myristic acid in total lipids of REF52 cells

Cellular lipids	Incorporation (% of total ^{3}H)
Lysophosphatidylcholine	0.5
Sphingomyelin	2.0
Phosphatidylcholine	82
Inositol plus serine phospholipids	1.0
Phosphatidylethanolamine	4.0
Monoacylglycerol	1.0
Diacylglycerol	0.9
Fatty acid	0.5
Triacylglycerol	6.0
Cholesterol ester	1.0

Cells were labeled with [^{3}H]myristic acid (0.5 μCi/ml) for 60 min, and total lipids were extracted, resolved, and analyzed for tritium as detailed in section 2. Data represent the mean of triplicate cultures

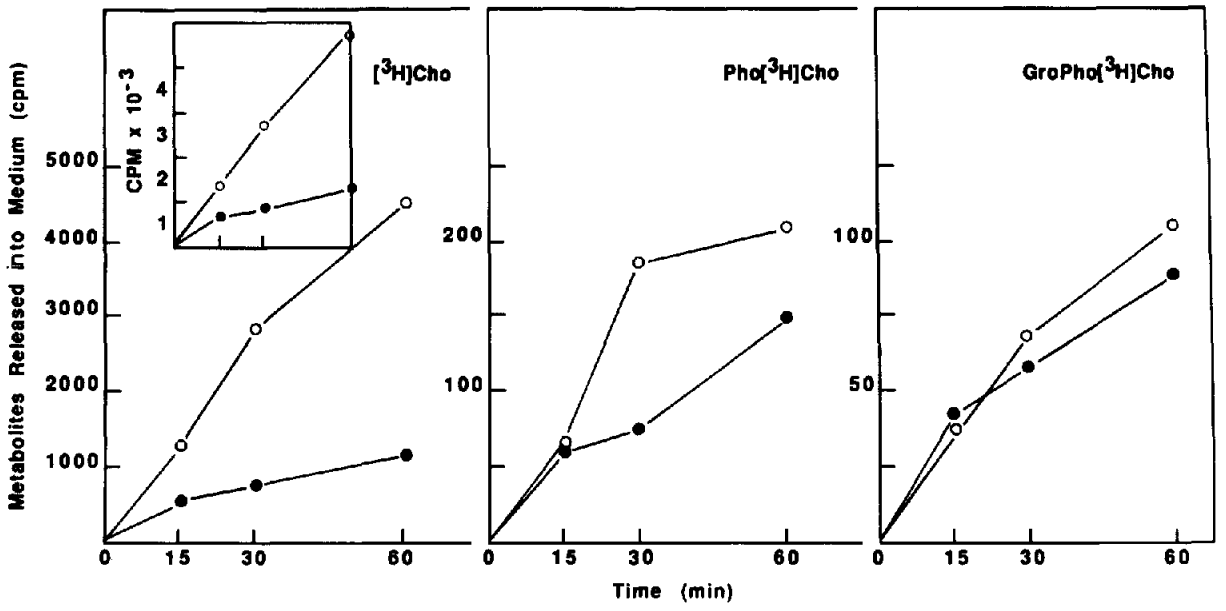

Fig.1. TPA elicits a time-dependent release of choline in REF52 cells. Cultures were prelabeled with [^{3}H]choline and incubated in the absence (●) or presence of TPA (○), 8×10^{-8} M, for the times shown. The tritium released into the medium was analyzed as described in section 2.3. Inset shows total water-soluble radioactivity released. Incubations were performed in duplicate; repeated experiments gave identical results. Cho, choline; PhoCho, phosphocholine; GroPhoCho, glycerophosphocholine.

acid were used to assay the evolution of [^{3}H]myristoyl-PA.

The data in table 2 demonstrate that two agonists, which stimulate PKC directly, elicit the formation of [^{3}H]myristoyl-PA. TPA and DiC8 treatment caused a 5.2- and 6.7-fold increase in [^{3}H]PA, respectively. TPA and DiC8 treatment also stimulated a 2.5- and 2.0-fold increase in DG (table 2). Treatment with VP caused similar responses. The temporal pattern of TPA-induced PC hydrolysis is illustrated in fig.2. The data (fig.2, inset) show that PC hydrolysis is accompanied by the initial formation of PA, and the formation of DG follows. Fig.2 shows that PA and DG radioactivity in untreated cultures was relatively stable throughout the 15-min incubation period. However, in TPA-exposed cells, the formation of [^{3}H]PA was rapid and exceeded that of DG at early times. The addition of unlabeled myristic acid (9 μM), either during the 60 min chase (post-labeling) or at the time of TPA addition, had no influence on the formation of [^{3}H]PA or [^{3}H]DG. The kinetics for the formation of [^{3}H]myristoyl-DG indicate that the DG is derived from PA. To verify this point, cells were preincubated with propranolol, a phosphatidate phosphohydrolase inhibitor [14]. Preliminary experiments showed that 100 μM propranolol was sufficient to block TPA-induced DG formation. The data in fig.3 show the time course for this response. Propranolol severely depressed the TPA-elicited production of DG, whereas PA generation proceeded.

Table 2

Agonist treatment elicits PC hydrolysis and formation of phosphatidate and diacylglycerols in REF52 cells

Cell treatment	cpm lipid ^{3}H/culture (± SD)	
	PA	DG
Control	1115 ± 35	3050 ± 90
TPA (8×10^{-8} M)	5770 ± 230	7605 ± 330
DiC8 (7.5×10^{-5} M)	7445 ± 140	6090 ± 315
Control	281 ± 48	1311 ± 59
VP (100 ng/ml)	731 ± 94	3182 ± 97

Cells, prelabeled with [^{3}H]myristic acid, were incubated ± agonists for 10 min. Lipids were analyzed as described in section 2. $n = 3$ for the TPA and DiC8 series; $n = 5$ for the VP experiments. The VP experiments were carried out in smaller culture dishes, hence the lower labeling values

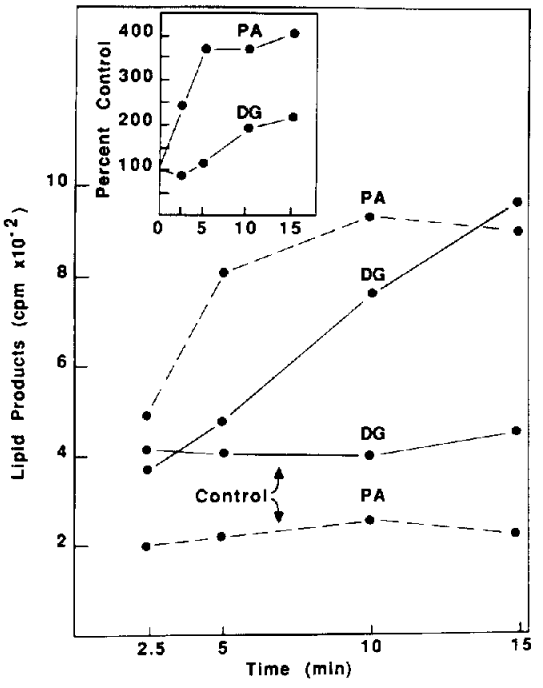

Fig.2. TPA-induced PC hydrolysis proceeds via rapid formation of phosphatidate and subsequent generation of diacylglycerol. Cultures were prelabeled with [^{3}H]myristic acid and incubated in the absence of (control) or presence of TPA (8×10^{-8} M) for the times shown. Data points are cpm/culture and are the average of triplicate cultures (SD < 10% of mean). Data from three other experiments gave similar results.

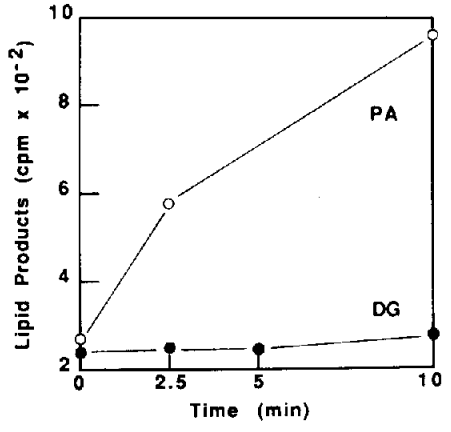

Fig.3. The influence of propranolol on TPA-induced PC degradation in REF52 cells. Cultures were prelabeled with [^{3}H]myristic acid and preincubated with propranolol (100 μM, 3 min) before the addition of TPA (8×10^{-8} M) for the times indicated. Cellular [^{3}H]PA and [^{3}H]DG were analyzed as described (section 2.3). Data points are the averages of 2–3 cultures (variation < 10%); repeated experiments gave similar results.

VP-induced PC hydrolysis, assessed by [^{3}H]choline and [^{3}H]myristic acid labeling techniques, was similar to that evoked by TPA. With VP treatment, the release of [^{3}H]choline and formation of intracellular [^{3}H]PA occurred early on (measurable at 1 min) and [^{3}H]DG formation followed (not shown).

4. DISCUSSION

Phorbol diester-induced PC degradation has been suggested to occur via the action of cellular phospholipase C [7,8,15,16]. In REF52 cells we have demonstrated that phospholipase D is the initial lipolytic event in TPA-induced breakdown of PC. A phospholipase D-related pathway for PC hydrolysis was suggested in the work of Liscovitch et al. [17] who found a preponderance of choline release in NG108-15 cells treated with TPA. The relationship of the two pathways of PC degradation, the phospholipase D pathway and the phospholipase C pathway is presently not known. As suggested previously [9], it is possible that the importance of a particular phospholipase is a function of the cell type and/or agonist used. In work similar to our TPA studies, Bocckino et al. [18] have shown that phosphatidate accumulation in hormone-treated hepatocytes occurs via a phospholipase D mechanism. Our data with VP action on REF52 cells are in agreement. In work submitted (Cabot, M.C., Welsh, C.J., Zhang, Z. and Cao, H., Evidence for a protein kinase C-directed mechanism in agonist-induced diacylglycerol generation from phosphatidylcholine), we have shown evidence that PC degradation in REF52 cells occurs via a PKC-directed mechanism.

Most of what is known of mammalian phospholipase D comes from the work of Witter and Kanfer [19]. Our results which implicate a phospholipase D pathway for PC degradation are based on: (i) cellular release of [^{3}H]choline; (ii) rapid intracellular formation of [^{3}H]PA followed by [^{3}H]DG; (iii) blocking of [^{3}H]DG formation by propranolol. Others have shown, as we have, that phospholipase D is hormone-inducible [9,18]. It is interesting to note that agonist-induced PC hydrolysis is severely depressed or totally absent in several transformed derivatives of the parent REF52 cell line [9]. As the action of phospholipase

D results in the formation of PA, a proported second messenger [20,21], and DG, a known activator of PKC [3], it is tempting to speculate that phospholipase D plays a role in cellular regulation. In addition to REF52 cells we have recently demonstrated a similar VP-induced phospholipase D pathway in vascular smooth muscle cells [22], and it is in smooth muscle cells that PA is thought to function as a second messenger [20].

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