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Evidence for a protein kinase C-directed mechanism in the phorbol diester-induced phospholipase D pathway of diacylglycerol generation from phosphatidylcholine

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In this study we provide evidence for the involvement of protein kinase C (PKC) in phorbol diester-induced phosphatidylcholine (PC) hydrolysis by the phospholipase D pathway. Rat embryo fibroblasts (REF52) were prelabeled with either tritiated choline or myristic acid; these compounds are preferentially incorporated into cellular PC. Phorbol diester-induced PC degradation was determined by measuring the release of [^{3}H]choline, and the formation of [^{3}H]myristoyl-containing phosphatidate (PA), diacylglycerol (DG), and phosphatidylethanol (PE). Staurosporine, a PKC inhibitor, blocked from 73 to 90% of the phorbol diester-induced PC hyrolysis. The inhibition of phorbol diester-induced choline release by staurosporine was dose dependent with an approximate ED_{50} of 150 nM. Pretreatment of cells with phorbol diester inhibited subsequent phorbol diester-induced PC degradation by 78–92%. A close correlation between the ED_{50} for phorbol diester-stimulated choline release and the K_d for phorbol diester binding was demonstrated. Neither forskolin nor dibutyryl cAMP elicited cellular PC degradation. In vitro experiments using phospholipase D from *Streptomyces chromofuscus* showed that staurosporine did not inhibit and TPA did not stimulate enzyme activity.

Protein kinase C; Phospholipase D; Phorbol diester; Phosphatidylcholine; Diacylglycerol

1. INTRODUCTION

The classical pathway for agonist-induced generation of cellular DG is via the hydrolysis of polyphosphoinositides by phospholipase C [1]. This early hormone- and growth factor-elicited event results in the liberation of DG second messengers that stimulate PKC [2]. Use of the tumor promoter, TPA, has facilitated the study of biological events downstream from hormone and growth factor receptor-mediated events. This is because TPA interacts directly with PKC [3,4] and thereby circumvents the initial lipid metabolic steps that are a characteristic of cellular responses to a variety of natural agonists. Several laboratories have reported on a PC pathway of DG production in cultured cells [5–11]. The discovery of this pathway is of interest because it provides an additional source of lipid mediators from non-phosphoinositide stores.

It is often assumed that TPA provokes changes in lipid metabolism through a PKC-related mechanism. Using PKC inhibitors, Parker et al. [12] have shown that PKC is involved in phorbol ester-stimulated arachidonic acid release. In an effort to elucidate the mechanisms and biological

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Abbreviations: DG, diacylglycerol; PKC, Ca^{2+}-activated, phospholipid-dependent protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; PC, phosphatidylcholine; PDBu, phorbol dibutyrate; Bu_2cAMP, 2′-*O*-dibutyryl-adenosine 3′,5′-cyclic monophosphate; BSA, bovine serum albumin; Me_2SO, dimethyl sulfoxide; DiC8, dioctanoyl glycerol; PBS, phosphate-buffered saline; TLC, thin-layer chromatography; IP_3, *myo*-inositol 1,4,5-trisphosphate; PE, phosphatidylethanol. In the naming of PC, PA, DG and PE, we do not distinguish between the type of aliphatic linkage to glycerol (ester, ether)

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significance of the PC degradation pathway, we have been investigating this response in a cultured rat embryo-derived cell line, REF52. Our previous findings demonstrate that DG generated during TPA treatment originates from cellular PC [10] by a pathway involving activation of phospholipase D [13]. We have, in this study, investigated the participation of PKC in phorbol diester-induced hydrolysis of cellular PC by the phospholipase D pathway. The data demonstrate that staurosporine, a PKC inhibitor [14], and down-regulation of PKC were dually effective in suppressing TPA-induced PC degradation. These results provide evidence that PKC mediates phorbol diester induction of PC hydrolysis by phospholipase D.

2. MATERIALS AND METHODS

2.1. *Materials*

[9,10(N)-^{3}H]Myristic acid (20–40 Ci/mmol) was purchased from New England Nuclear and Amersham. [*methyl*-^{3}H]Choline chloride (80 Ci/mmol), L-*myo*-[1,2-^{3}H(N)]inositol (60 Ci/mmol) and [20-^{3}H(N)]PDBu (10–20 Ci/mmol) were purchased from New England Nuclear. TPA and PDBu were supplied by Chemicals for Cancer Research (Eden Prairie, MN). Phospholipase C (*B. cereus*), phospholipase D (*S. chromofuscus*) and Bu_2cAMP were from Sigma. Neutral lipids and phospholipids were purchased from Nu Chek Prep (Elysian, MN) and Avanti Polar Lipids (Birmingham, AL), respectively. Silica gel G TLC plates were from Analtech (Newark, DE) and FBS was purchased from HyClone (Logan, UT). Forskolin was purchased from Calbiochem. Staurosporine was kindly provided by Dr Hiroshi Kase (Kyowa Hakko Kogyo Co., Ltd, Tokyo Research Laboratories, Tokyo).

2.2. *Cell radiolabeling, treatment, and enzyme assay*

REF52 cells were cultured and prelabeled with [^{3}H]myristic acid (1 or 24 h) or [^{3}H]choline (24 or 48 h) as described in [10,11,13]. When cells were prelabeled for 24 h with [^{3}H]myristic acid, medium containing 5% FBS was used. TPA (8×10^{-8} M) was added as detailed [10]. Staurosporine (2.0 mM solution, Me_2SO) was diluted with medium to achieve the concentrations desired; Me_2SO was present in control cultures. Experiments were carried out in serum-free medium containing BSA [13]. For pre-exposure to phorbol diesters, cells were cultured in 5% FBS medium ± PDBu (1×10^{-6} M) and [^{3}H]choline for 24 h. After removal of isotope, cultures were equilibrated as in [13] before initiating experiments. DiC8 was prepared by phospholipase C (*B. cereus*) digestion [10] of dioctanoylphosphatidylcholine. PE formation was assessed using cells prelabeled with [^{3}H]myristic acid (24 h) and medium containing 0.7% ethanol during the treatment period. The influence of TPA and staurosporine on in vitro phospholipase D (*S. chromofuscus*) activity was also evaluated; the assays [15] employed phosphatidyl[^{14}C]choline (2.0 mM), synthesized as detailed [10] and diluted with egg PC (spec. act. 470 dpm/nmol). The incubations (10–15 min, 5–20 U enzyme/ml) were terminated by addition of solvents [16], and [^{14}C]choline was quantitated as described [10].

Bu_2cAMP (1.0 mM) was tested alone or in combination with TPA (30 min at 37°C) using cells prelabeled with [^{3}H]choline. The influence of forskolin (Me_2SO vehicle) on PC degradation was examined in cells prelabeled with [^{3}H]choline and [^{3}H]myristic acid (separate experiments). Cultures were incubated for 15 min at 37°C (± forskolin) and PC metabolism was analyzed as detailed above. PDBu specific binding was determined as described [17]. Phosphoinositide degradation was measured [18] after cells had been labeled for 48 h with [^{3}H]inositol (10 μCi/ml 10% FBS medium).

2.3. *Lipid analysis*

Total cellular lipids were extracted [16] as previously detailed [10]. Phosphatidate (dioleoyl) carrier (25 μg) was added to each extraction. Radiolabeled DG and phosphatidate were resolved from total lipids using the solvent systems described in [10,13]. PE was resolved from total cellular lipids by TLC in a solvent system containing chloroform/methanol/NH_4OH (65:30:3, v/v). Lipids were visualized using iodine [10,13], and radioactivity was determined by liquid scintillation spectrometry. [^{3}H]Choline released into the culture medium was determined as in [13,19].

3. RESULTS

TPA and DiC8, agents known to stimulate PKC [3,4,20], were employed in initial experiments to investigate the relationship between PKC activation and PC hydrolysis by the phospholipase D pathway. In cells labeled with [^{3}H]myristic acid, 10 min treatment with TPA (8×10^{-8} M) or DiC8 (7.5×10^{-5} M) elicited an approx. 5- and 7-fold increase over control in [^{3}H]PA formation, respectively. This was accompanied by a 2–2.5-fold increase in [^{3}H]DG. Additional experiments revealed that radioactivity in PC was diminished by 18%, compared to control cultures (control, 6598 cpm ± 200; TPA, 5417 cpm ± 183; $P < 0.0001$), when cells were treated with TPA for 1 h. The dose-response relationship for phorbol diester-induced choline release and the binding of phorbol diesters to REF52 cells were also evaluated. The data in fig.1 show that increasing the concentration of PDBu causes an increase in the release of [^{3}H]choline ($ED_{50} \sim 28$ nM). The K_d for PDBu binding to REF52 cells was ~32 nM (personal communication, Dr Susan Jaken, W. Alton Jones Cell Science Center).

Protocols commonly used to attenuate PKC activity were employed to examine further the

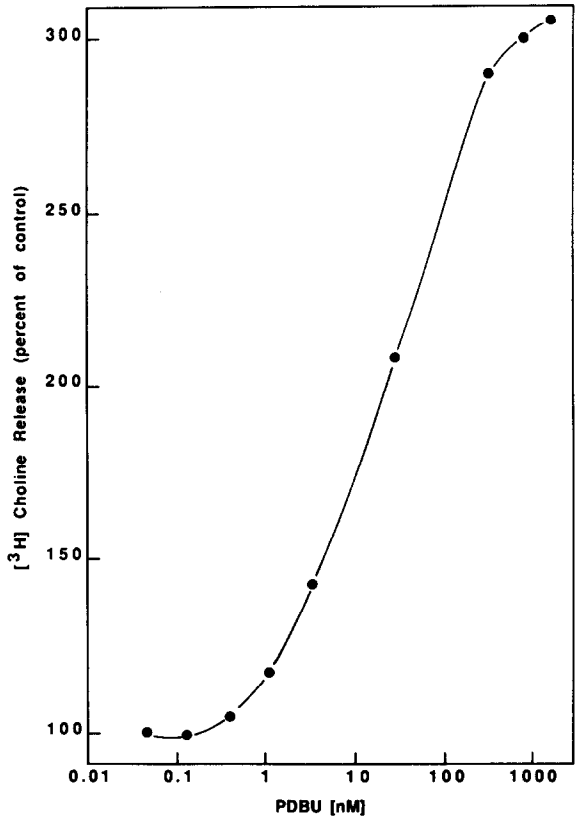

Fig.1. Dose-response relationship for phorbol diester-induced PC hydrolysis by phospholipase D in REF52 cells. Cultures were labeled with [^{3}H]choline (1.0 μCi/ml medium) for 24 h, equilibrated as described in section 2, and exposed to increasing concentrations of PDBu for 60 min. All cultures contained Me_2SO (0.02%). [^{3}H]Choline released to the medium was quantitated as described in the text. Results represent means ± SD (n = 3 for each point); variances were < 10% of the mean. Repeated experiments gave similar results.

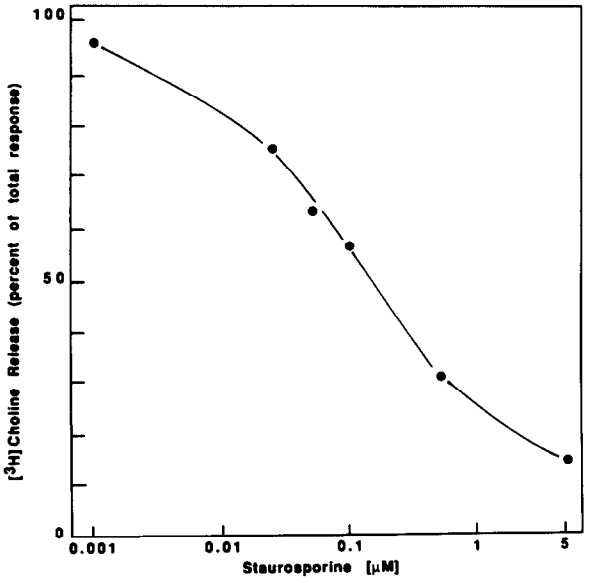

Fig.2. The inhibitory influence of staurosporine on phorbol diester-induced PC degradation by the phospholipase D pathway in REF52 cells. Cultures were prelabeled with [^{3}H]choline, equilibrated, incubated in the absence or presence of staurosporine at the concentrations indicated, and exposed to TPA (20 min) as described in the text. Variances were < 5% of the mean; n = 3 for each point (± SD). Total response in the absence of staurosporine is 100%.

association between PKC and phospholipase D. Fig.2 shows that staurosporine, a PKC inhibitor [14], blocked TPA-induced [^{3}H]choline release in a dose-dependent fashion (ED_{50} ~ 150 nM). The effect of staurosporine on TPA-induced PC hydrolysis was also measured by (in addition to choline release) the generation of two direct hydrophobic degradation products of phospholipase D, PA and PE, and formation of one indirect product, DG. The data in table 1 show that staurosporine inhibited TPA-induced PC hydrolysis as measured by all four parameters. TPA caused a 2.1-fold increase in choline release, whereas staurosporine decreased this to near control values (inhibition of ~90%). Concomitant with this response, staurosporine inhibited TPA-induced PA formation by ~76% and phospholipase D-catalyzed PE synthesis by ~91%. The production of DG was likewise inhibited by staurosporine (90%).

Experiments were conducted using down-regulation protocol, a valuable technique for assessing PKC-mediated cellular events. The data in table 2 show that pretreatment of cells with PDBu largely inhibited subsequent phorbol diester-induced choline, PA, and DG formation. In cultures preexposed to PDBu, phorbol diester-induced [^{3}H]choline release was inhibited by ~86%, and [^{3}H]PA formation was reduced by ~78%. The generation of [^{3}H]DG was likewise inhibited (~92%).

The effects of staurosporine and phorbol diesters were examined on several other enzymatic responses. Vasopressin, which induces PC hydrolysis in REF52 cells [10,11,13], also elicits polyphosphoinositide hydrolysis in this cell line.

Table 1

The influence of the PKC inhibitor, staurosporine, on TPA-induced PC hydrolysis in REF52 cells: inhibited formation of hydrophilic and hydrophobic degradation products

Cell treatment	PC hydrolysis products (cpm metabolites/culture)			
	Hydrophilic	Hydrophobic		
	[^{3}H]Choline	[^{3}H]PA	[^{3}H]DG	[^{3}H]PE
Control	2021 ± 195	1890 ± 120	3035 ± 115	1200 ± 140
TPA	4212 ± 136	6550 ± 210	4910 ± 275	3685 ± 145
Staurosporine	1859 ± 172	1690 ± 120	3260 ± 115	1590 ± 105
Staurosporine + TPA	2057 ± 183	2805 ± 255	3480 ± 145	1820 ± 195

Cells were prelabeled with either [^{3}H]choline or [^{3}H]myristic acid as described in section 2, and then preincubated in control (0.25% Me_2SO) or staurosporine (5×10^{-6} M) medium for 3 min at room temperature. TPA (8×10^{-8} M) was added, and cells were further incubated at 37°C for 30 min ([^{3}H]choline), 10 min ([^{3}H]myristoyl-PA; [^{3}H]myristoyl-DG) or 15 min ([^{3}H]myristoyl-PE). PC hydrolysis products were determined as described in the text. All values shown are mens ± SD (n = 3) for each experimental group. [^{3}H]PA and [^{3}H]DG were analyzed from the same experiment; [^{3}H]choline and [^{3}H]PE data represent separate experiments

Vasopressin (100 ng/ml, 30 s) caused a 6.5-fold increase in the amount of [^{3}H]IP_3 (not shown). Under identical conditions, staurosporine (5 μM) had no influence on vasopressin-induced [^{3}H]IP_3 formation. Staurosporine (5 μM) did not modify the hydrolysis of phosphatidyl[^{14}C]choline when assayed in vitro using phospholipase D from *S. chromofuscus*. Lastly, to determine whether TPA interacted directly with phospholipase D, in vitro assays using enzyme from *S. chromofuscus* were conducted; over the concentration range tested (3×10^{-8}–8×10^{-7} M), TPA had no influence on enzyme activity.

The possible participation of an adenylate cyclase/cAMP mechanism in PC degradation was examined. In cells prelabeled with [^{3}H]choline,

Table 2

Pretreatment of REF52 cells with PDBu inhibits phorbol diester-induced PC degradation by the phospholipase D pathway

Agonist	PDBu pretreatment	PC hydrolysis products (cpm metabolites/culture)		
		[^{3}H]Choline	[^{3}H]PA	[^{3}H]DG
Control	–	1368 ± 115	4365 ± 190	3655 ± 135
Phorbol diester	–	3996 ± 105	12150 ± 570	6440 ± 495
Control	+	1531 ± 79	4665 ± 150	3645 ± 35
Phorbol diester	+	1939 ± 38	6675 ± 210	3865 ± 135

Cells were prelabeled with either [^{3}H]choline or [^{3}H]myristic acid, in the absence or presence of PDBu (1×10^{-6} M) for 24 h. [^{3}H]Choline-labeled cells were challenged with PDBu (8×10^{-7} M) for 30 min, and cultures labeled with [^{3}H]myristic acid were challenged with TPA (8×10^{-8} M) for 10 min at 37°C. The presence of PDBu during the prelabeling period did not alter uptake or incorporation of isotopes (vs controls). PC hydrolysis products, [^{3}H]choline, [^{3}H]myristoyl-PA and [^{3}H]myristoyl-DG, were determined as described in the text. All values are means ± SD (n = 3) for each experimental group. [^{3}H]PA and [^{3}H]DG were analyzed from the same experiment; similar results were obtained using PDBu as agonist

Bu_2cAMP (1.0 mM, 30 min treatment) elicited only a minor increase in the release of [^{3}H]choline-containing compounds to the medium (21% above control). The influence of Bu_2cAMP, added together with TPA, did not increase or decrease cellular phospholipase D activity over that elicited by TPA alone. Exposure of cells to forskolin (5–25 μM, 15 min treatment) did not cause an increase in the release of [^{3}H]choline metabolites over control levels, nor did forskolin elicit intracellular formation of [^{3}H]PA or [^{3}H]DG over control values (not shown).

4. DISCUSSION

Our results implicating PKC involvement in the phospholipase D pathway of PC degradation are based on: (i) elicitation of PC hydrolysis by PKC agonists; (ii) a close correlation between the ED_{50} for choline release and the K_d for phorbol diester binding; (iii) impedance of phorbol diester-induced PC degradation by the PKC inhibitor, staurosporine; (iv) inhibition of phorbol diester-induced PC degradation by cellular pretreatment with phorbol diesters. Phospholipase D activity was also evaluated by the formation of intracellular PE; in the presence of ethanol the enzyme catalyzes a transphosphatidylation reaction to produce PE [21,22]. By using several techniques to measure intracellular PC degradation, our results show that methods employed to block PKC action also elicit diminished phospholipase D activity. Staurosporine did not modify the action of phospholipase D (*S. chromofuscus*) when assayed in vitro. Furthermore, although we have no definitive data to dispel the notion that phorbol diesters stimulate intracellular phospholipase D directly, experiments employing phospholipase D from *S. chromofuscus* showed that TPA did not modify enzyme activity.

Hormone-induced accumulation of PA by a phospholipase D mechanism has been demonstrated in hepatocytes [22]; the response could be activated, in vitro, by the addition of guanine nucleotides. As phorbol diesters circumvent cellular transducing elements, our data would indicate that TPA, via interaction with PKC, stimulates phospholipase D activity by a mechanism that does not require an initial G-protein-coupled reaction. Similar conclusions were made for agonist-induced PE synthesis in HL-60 cell lysates [23]. We have also shown that the PKC inhibitor, staurosporine, does not modify the G-protein-requiring pathway of vasopressin-induced PIP_2 degradation in REF52 cells.

Our data exemplify the diversity of biochemical responses elicited by the PKC family of isozymes [24,25]. A role for phospholipase D in agonist-induced cellular events was suggested, early on, in the work of Hokin-Neaverson and co-workers [26]. In the light of recent research, we postulate that the action of phospholipase D constitutes a key element in PA- and DG-governed cellular responses, and as such, phospholipase D could play an important role in the physiological regulation of agonist-induced events.

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