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Stimulation of phosphatidylcholine synthesis by activators of protein kinase C is dissociable from increased phospholipid hydrolysis

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The aim of this study was to clarify the relationship between the stimulatory effects of protein kinase C activators, including phorbol 12-myristate 13-acetate (PMA) and bryostatin, on the hydrolysis of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) and on PtdCho synthesis. The cell lines used were selected because of their differential responses to protein kinase C activators and included rat-1 fibroblasts, untransformed and *A-raf*-transformed NIH 3T3 fibroblasts and human HL60 leukaemia cells. Exposure of rat-1 and NIH 3T3 fibroblasts to 100 nM-PMA stimulated phospholipase D-mediated hydrolysis of phospholipids about 2- and 6-fold respectively. In contrast, 100 nM-PMA had similar (2.5–3.0-fold) stimulatory effects on PtdCho synthesis in these cell lines. In the untransformed NIH 3T3 cells, both PMA and bryostatin stimulated both phospholipid hydrolysis and PtdCho synthesis, with 100 nM-bryostatin being somewhat less potent than 100 nM-TPA. In contrast, in *A-raf*-transformed NIH 3T3 cells or in HL60 cells, only TPA, but not bryostatin, stimulated PtdCho synthesis. In these transformed cells, bryostatin had 3-fold, or higher, stimulatory effects on phospholipid hydrolysis. Addition of ionomycin, a Ca^{2+}-elevating agent, partially restored the stimulatory effect of bryostatin on PtdCho synthesis, but it failed to modify the effect of bryostatin on phospholipid hydrolysis. These data indicate that increased phospholipid hydrolysis is not necessarily associated with increased PtdCho synthesis.

INTRODUCTION

An important function of activated protein kinase C is the regulation of phosphatidylcholine (PtdCho) synthesis. It is well established that activators of protein kinase C, such as the potent tumour promoter phorbol 12-myristate 13-acetate (PMA) [1], stimulate the formation of CDP-choline [2–5], the rate-limiting substrate for PtdCho synthesis [6–10]. Since activators of protein kinase C failed to enhance the phosphorylation of choline-phosphate cytidylyltransferase (EC 2.7.7.15) in intact cells [5] or in assays *in vitro* [11,12], the activity of cytidylyltransferase may be regulated by PMA and other protein kinase C activators through an indirect mechanism(s).

In most cell types studied, activators of protein kinase C also stimulate phospholipase D-mediated hydrolysis of PtdCho [13] and phosphatidylethanolamine (PtdEtn) [14,15]. 1,2-Diacylglycerol (1,2-DAG), a possible degradation product, is known to stimulate PtdCho synthesis [16,17]. Treatment of various cells with phospholipase C enhanced PtdCho synthesis by provoking redistribution of cytidylyltransferase from the cytosol to internal membranes [18–22]. Since phospholipase C attacks the outer surface of the plasma membrane, it is difficult to understand how such redistribution of cytidylyltransferase takes place. However, available evidence strongly suggests that 1,2-DAG, generated by exogenous phospholipase C, induces redistribution of cytidylyltransferase [20,21]. It has been suggested that, by analogy with the action of phospholipase C, a similar causal relationship between PMA-induced phospholipid hydrolysis and PtdCho synthesis involving 1,2-DAG as a mediator may exist [11,23–25].

The aim of this study was to determine a possible correlation between phospholipid hydrolysis and PtdCho synthesis in fibroblasts and HL60 leukaemia cells treated with various activators of protein kinase C. The data presented are inconsistent with a direct role of phospholipid hydrolysis in the regulation of PtdCho synthesis.

EXPERIMENTAL

Materials

PMA, Dowex-50-W (H^+ form), phospholipase C (type IX; from *Clostridium perfringens*), phospholipase A_2 (from bee venom), phospholipase B (from *Vibrio* species), PtdCho, PtdEtn, phosphatidic acid (PtdOH) and 1,2-DAG were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; [2-^{14}C]ethanolamine (20 mCi/mmol), [1-^{14}C]palmitic acid (60 mCi/mmol) and [*methyl*-^{14}C]choline chloride (50 mCi/mmol) were from Amersham, Arlington Heights, IL, U.S.A.; and tissue-culture reagents were brought from GIBCO, Grand Island, NY, U.S.A. Bryostatin 1 was purified from the marine animal *Bugula neritina* [26]. Phosphatidylethanol was prepared by a phospholipase D-catalysed reaction from PtdCho and ethanol [27].

Cell culture

Rat-1 fibroblasts, NIH 3T3 clone-7 untransformed fibroblasts and NIH 3T3 clone-7 fibroblasts transformed with human *A-raf* (NIH 3T3/*A-raf*) [28] were provided by Dr. Ulf R. Rapp (National Cancer Institute, Frederick, MD, U.S.A.) and were continuously cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-calf serum, penicillin (50 units/ml)/streptomycin (50 μg/ml) and glutamine (2 mM). Fibroblasts were seeded [$(1–2) \times 10^5$/dish] in 100 mm-diam. plastic dishes (Costar), and growing (50–70% confluent) cell populations were harvested after 2 days in culture. The human promyelocytic-leukaemia cell line HL60 [29] was continuously

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdOH, phosphatidic acid; 1,2-DAG, 1,2-diacylglycerol; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; diC_8, 1,2-dioctanoyl-*sn*-glycerol.

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cultured in RPMI 1640 medium supplemented with 20% heat-inactivated fetal-calf serum, penicillin (50 units/ml)/streptomycin (50 μg/ml) and glutamine (2 mM). Cells were harvested for experiments at a density of $(0.8–1.0) \times 10^6$/ml.

Measurement of PMA-stimulated hydrolysis of PtdCho and PtdEtn

Attached NIH 3T3 cells were incubated with either [*methyl*-^{14}C]choline (0.20 μCi/ml) or [2-^{14}C]ethanolamine (0.15 μCi/ml) for 48 h, washed, incubated for 3 h in the presence of fresh medium, and then harvested by gently scraping from the dish. Collected cells, pooled from 3–5 dishes (150 cm^2), were washed. Then 0.3 ml portions of cell suspension [$(1.2–2.8) \times 10^6$ cells/ml] were incubated (final vol. 0.32 ml) in polypropylene tubes in an incubator at 37 °C in the presence of 20 mM unlabelled choline or 2 mM-ethanolamine, as indicated, along with other agents as specified. Incubations were terminated by addition of 4 ml of chloroform/methanol (1:1, v/v). Cells were extracted in this mixture for 60 min at room temperature, followed by addition of 3 ml of water to initiate phase separation. After centrifuging the samples for 5 min at 2000 ***g***, samples of the water/methanol phase were used for determination of total released ^{14}C radioactivity, as well as for the separation of choline and ethanolamine metabolites. For this latter purpose, essentially the method described by Cook & Wakelam [30] was used. Briefly, Dowex-50-W (H$^+$) packed columns (Bio-Rad Econo-columns; 1 ml bed vol.), treated with 1 M-HCl for 2 h, were washed with water until the washings were approx. pH 5.5. The samples were diluted to 5 ml with distilled water and loaded on to the columns. The initial flow-through along with a following 5 ml water wash contained either [^{14}C]glycerophosphocholine or [^{14}C]glycerophosphoethanolamine. [^{14}C]Choline phosphate or [^{14}C]ethanolamine phosphate was eluted by 20 ml of water, and [^{14}C]choline or [^{14}C]ethanolamine was eluted by 20 ml of 1 M-HCl. Each fraction was evaporated to dryness, and their ^{14}C content was determined by liquid-scintillation counting. For the preparation of appropriate standards, the ^{14}C-labelled cells were treated with either phospholipase A_2 (1 unit/ml) + phospholipase B (1 unit/ml) (to produce glycerophospho-bases), or phospholipase C (1 unit/ml) (to produce phosphorylated bases), for 60 min, and extracts were prepared as above. For determination of the elution profiles of standards (determined in each experiment), 2 ml fractions were collected. The metabolites of [^{14}C]choline and [^{14}C]ethanolamine present in the peak fractions were further identified by t.l.c. [14]. Phospholipids, present in the lower chloroform phase, were separated, and their ^{14}C content was determined as described above. In typical experiments, the ^{14}C content of PtdEtn and PtdCho pools was 168000–184000 and 139000–155000 d.p.m./10^6 cells respectively.

Measurement of PtdCho synthesis in intact cells

For the measurement of PtdCho formation, fibroblasts were harvested as above. Fibroblasts [$(1.2–1.6) \times 10^6$/ml)] or HL60 cells (1.5×10^6/ml) were suspended in fresh Dulbecco's medium or RPMI 1640 medium respectively, and incubated with either [*methyl*-^{14}C]choline (1 μCi/ml) or [^{32}P]P_i (0.3 mCi/ml) for 20–120 min in the presence of agents as indicated. Incubations were terminated by addition of chloroform/methanol (1:1, v/v). PtdCho was separated from the other lipids by silica-gel t.l.c., and the radioactivity in PtdCho was determined [31,32].

Measurement of formation of PtdOH, phosphatidylethanol and 1,2-DAG in fibroblasts prelabelled with [^{14}C]palmitic acid

Cells were prelabelled with [1-^{14}C]palmitic acid (0.25 μCi/ml) for 24 h. Cells were washed, incubated in fresh medium for an additional 2 h (to minimize the amount of unesterified labelled palmitic acid), and then harvested from 3–4 dishes (150 cm^2). Cells were washed and 0.3 ml portions [$(1.6–2.4) \times 10^6$ cells] were incubated (final vol. 0.32 ml) with PMA and other agents as indicated for 20–30 min. Incubations were terminated by addition of 4 ml of chloroform/methanol (1:1, v/v), and neutral lipids (present in the chloroform phase) were separated by silica-gel t.l.c. in the solvent system hexane/diethyl ether/acetic acid (70:30:2, by vol.). PtdOH was separated from other phospholipids as described previously [31,32]. Phosphatidylethanol was separated by silica-gel t.l.c. by using the organic phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10, by vol.) as the developing solvent [33].

Determination of intracellular content of 1,2-DAG

This was done after its quantitative conversion into PtdOH, by using 1,2-DAG kinase and [γ-^{32}P]ATP as described by Preiss *et al.* [34].

RESULTS

Effects of PMA on PtdCho synthesis and phospholipid hydrolysis in NIH 3T3 and rat-1 fibroblasts

A possible quantitative relationship between the stimulatory effects of PMA on phospholipid hydrolysis and PtdCho synthesis was initially studied in NIH 3T3 and rat-1 fibroblasts. In agreement with previous data [14,15], PMA was a potent stimulator of phospholipase D-catalysed hydrolysis of the ^{14}C-prelabelled pools of PtdCho and PtdEtn in NIH 3T3 cells (Table 1). In contrast, PMA was a less potent stimulator of phospholipid hydrolysis in rat-1 fibroblasts (Table 1). Thus, although the treatment of ^{14}C-prelabelled rat-1 cells with 100 nM-PMA for 1 h resulted in the hydrolysis of only 3.3–3.4% of the total cellular pools of PtdCho and PtdEtn, a similar treatment of NIH 3T3 cells with PMA resulted in the hydrolysis of 11.3–13.6% of these phospholipids.

Another widely used method to measure phospholipase D activity is based on the ability of this enzyme to catalyse a transphosphatidylation reaction. In this reaction the phosphatidyl group of PtdCho or PtdEtn is transferred to ethanol or other alcohols [35]. The resulting product is a phosphatidylalcohol, which, unlike PtdOH, is only slowly metabolized in cells [35]. As shown in Table 2, formation of phosphatidylethanol in

Table 1. Effect of PMA on phospholipase D-mediated hydrolysis of phospholipids in rat-1 and NIH 3T3 fibroblasts

Cells were prelabelled with [^{14}C]choline or [^{14}C]ethanolamine for 48 h, followed by incubation of cells for 60 min in the absence or presence of 100 nM-PMA as described in the Experimental section. The water-soluble ^{14}C-labelled products were separated by ion-exchange chromatography. The ^{14}C content of PtdCho in rat-1 and NIH 3T3 cells was 197000 and 178000 d.p.m./10^6 cells respectively. Data are means ± S.E.M. of four determinations. Similar results were obtained in two other experiments.

	Degradation of [^{14}C] phospholipids (% of total membrane pool of respective phospholipids)			
	[^{14}C]Choline		[^{14}C]Ethanolamine	
Addition	Rat-1	NIH 3T3	Rat-1	NIH 3T3
None	1.16 ± 0.12	1.90 ± 0.05	1.84 ± 0.07	2.43 ± 0.13
PMA (100 nM)	3.34 ± 0.15	11.31 ± 0.38	3.37 ± 0.14	13.62 ± 0.75

Table 2. Effect of PMA and ethanol on the formation of [^{14}C]phosphatidylethanol in rat-1 and NIH 3T3 fibroblasts

Cells were prelabelled with [^{14}C]palmitic acid for 24 h and then incubated in the absence or presence of 100 nM-TPA and/or 200 mM-ethanol for 20 min. The ^{14}C content of the total phospholipid fraction in rat-1 and NIH 3T3 cells was 135000 and 154000 d.p.m./10^6 cells respectively. Data are means ± S.E.M. of four determinations. Similar results were obtained in another experiment.

Addition	[^{14}C]Phosphatidylethanol (d.p.m./20 min per 10^6 cells)	
	NIH 3T3	Rat-1
None	0	0
PMA	0	0
Ethanol	0	0
PMA + ethanol	5470 ± 390	1420 ± 130

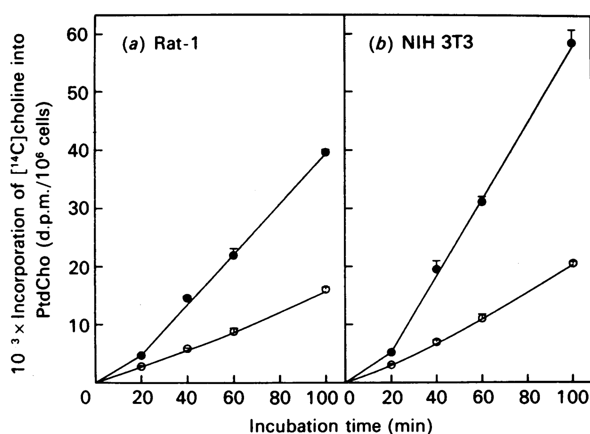

Fig. 1. Time course of PMA effect on the incorporation of [^{14}C]choline into PtdCho in rat-1 and NIH 3T3 fibroblasts

Cells were incubated with [^{14}C]choline for 20–100 min in the absence (○) or presence (●) of 100 nM-PMA. Each point represents the mean ± S.E.M. of four determinations. Similar results were obtained in another experiment.

[^{14}C]palmitate-prelabelled NIH 3T3 and rat-1 cells was observed only in the simultaneous presence of ethanol and PMA. Importantly, during a 20 min incubation period, stimulated NIH 3T3 cells converted about 3 times more ^{14}C-labelled phospholipid into phosphatidylethanol compared with stimulated rat-1 cells (Table 2).

The observed large differences in phospholipid hydrolysis in the two cell lines were not reflected in the effects of PMA on PtdCho synthesis. As shown in Fig. 1, treatment of rat-1 and NIH 3T3 fibroblasts with 100 nM-PMA for 100 min stimulated the incorporation of [^{14}C[choline into PtdCho in the rat-1 and NIH 3T3 cells 2.6- and 2.9-fold respectively. Similar results (not shown) were obtained when [^{32}P]P_i was used as the labelling agent, indicating that PMA-stimulated incorporation of [^{14}C]choline into phospholipid was due to increased PtdCho synthesis. In addition, (i) PMA had no effect on the uptake of [^{14}C]choline, (ii) it decreased the cellular level of [^{14}C]choline phosphate about 20%, and (iii) it stimulated the formation of [^{14}C]CDP-choline about 2.5-fold (results not shown). In agreement with data in the literature [2–5], our results are consistent with CDP-choline formation being the regulatory step for PtdCho synthesis in the PMA-treated fibroblasts.

Table 3. Effect of PMA on the formation of [^{14}C]PtdOH and [^{14}C]1,2-DAG in rat-1 and NIH 3T3 fibroblasts

Cells were prelabelled with [^{14}C]palmitic acid for 24 h and then incubated in the absence or presence of 100 nM-PMA for 30 min. The ^{14}C content of the total phospholipid fraction in rat-1 and NIH 3T3 cells was 286000 and 353000 d.p.m./10^6 cells respectively. Data are means ± S.E.M. of four determinations. Similar results were obtained in another experiment.

Addition	Formation (d.p.m./30 min per 10^6 cells)			
	[^{14}C]PtdOH		[^{14}C]1,2-DAG	
	NIH 3T3	Rat-1	NIH 3T3	Rat-1
None	7330 ± 310	5610 ± 520	3210 ± 200	2240 ± 180
PMA (100 nM)	11570 ± 240	4820 ± 390	4570 ± 280	1770 ± 260

Effects of PMA on the levels of 1,2-DAG and PtdOH in NIH 3T3 and rat-1 fibroblasts

Decreased phospholipid hydrolysis does not necessarily result in lower levels of 1,2-DAG, a possible mediator of PMA action on PtdCho synthesis. The effects of PMA on the cellular levels of 1,2-DAG were first examined in [^{14}C]palmitate-prelabelled NIH 3T3 and rat-1 fibroblasts. For comparative purposes, the relative levels of [^{14}C]PtdOH were also determined. Whereas in the NIH 3T3 cells a 30 min treatment with PMA enhanced the levels of both [^{14}C]PtdOH and, to a lesser extent, [^{14}C]1,2-DAG, in rat-1 cells PMA failed to increase either of the degradation products (Table 3). Using the 1,2-DAG kinase assay for the determination of 1,2-DAG levels [34], we also observed that treatment of NIH 3T3 cells with PMA for 30 min only minimally enhanced the cellular content of 1,2-DAG (1.25-fold), whereas a similar treatment of rat-1 cells actually decreased 1,2-DAG by 20% (results not shown). These data indicate that in the PMA-treated cells the synthesis of PtdCho does not correlate with the formation of 1,2-DAG. In addition, these results also suggest that in the PMA-treated cells 1,2-DAG is not the rate-limiting substrate for PtdCho synthesis.

Comparison of the effects of PMA and 1,2-DAG-elevating agents on PtdCho synthesis: inhibitory effects by H7

Since in intact cells PMA is the most efficient stimulator of protein kinase C-mediated protein phosphorylation [36–38], 1,2-DAG could mediate the effect of PMA on PtdCho synthesis only through a protein kinase C-independent mechanism. Therefore, we determined the role of protein kinase C in the mediation of 1,2-DAG and PMA effects on PtdCho synthesis by using H7, a commonly used inhibitor of this enzyme [39]. The cellular levels of 1,2-DAG were raised by using either diC_8, a cell-permeant 1,2-DAG analogue, or phospholipase C. Treatment of the [^{14}C]palmitate-prelabelled NIH 3T3 cells with this enzyme for 30 min resulted in a 5.5-fold increase in [^{14}C]1,2-DAG (results not shown). As shown in Table 4, treatment of NIH 3T3 cells with both phospholipase C and diC_8 enhanced, although with different efficiency, the synthesis of PtdCho. Importantly, the stimulatory effects of diC_8, phospholipase C and 100 nM-PMA were similarly inhibited by H7. We should note that H7 was a more efficient inhibitor of PMA action if added 20 min before PMA; however, the results also were more variable. In contrast, very reproducible effects were obtained at 300 μM concentration of H7. However, in the experiment shown in Fig. 2, even 300 μM-H7 failed to inhibit the effect of 3 nM-PMA on PtdCho synthesis. Significant inhibitory effects of H7 were evident only at higher

Table 4. Effect of PMA, diC$_8$, phospholipase C and H7 on PtdCho synthesis in NIH 3T3 cells

NIH 3T3 cells were incubated with [^{14}C]choline for 2 h in the absence or presence of PMA (100 nM), phospholipase C (0.2 unit/ml), diC$_8$ (50 μg/ml) and H7 (300 μM). Data are means $\pm$ S.E.M. of four determinations. Similar results were obtained in two other experiments.

Addition	Incorporation of [^{14}C]choline in PtdCho (d.p.m./2 h per 10^6 cells) None	H7 (300 μM)
None	13320 $\pm$ 390	13110 $\pm$ 420
PMA	40730 $\pm$ 1510	26260 $\pm$ 490
Phospholipase C	33880 $\pm$ 1140	17540 $\pm$ 630
DiC$_8$	18230 $\pm$ 360	15360 $\pm$ 280

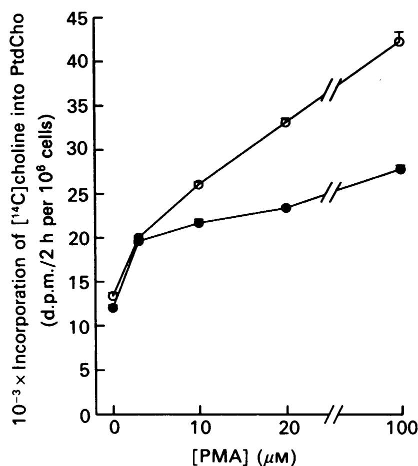

Fig. 2. Inhibition by H7 of PMA-stimulated PtdCho synthesis in NIH 3T3 cells

NIH 3T3 cells were incubated with [^{14}C]choline for 2 h in the presence of various concentrations of PMA without (○) or with (●) 300 μM-H7. Each point represents the mean $\pm$ S.E.M. of four determinations.

concentrations of PMA. In two other experiments, the effects of 2–5 nM-PMA also were unaffected by H7, whereas in a third experiment H7 even failed to inhibit the effect of 10 nM-PMA. However, in each experiment H7 inhibited the effect of 100 nM-PMA by at least 40%. Despite the slight variability of data we can conclude that the actions of lower and higher concentrations of PMA on PtdCho synthesis are differentially sensitive to the inhibitory effect of H7.

In another set of experiments, PMA, diC$_8$ or phospholipase C was added to NIH 3T3 cells pretreated with 300 nM-PMA for 24 h. Such prolonged treatment of fibroblasts with PMA causes down-regulation of protein kinase C [40–42]. In agreement with data from other laboratories [4,43], newly added PMA (100 nM) failed to stimulate PtdCho synthesis. In addition, pretreatment of cells with PMA also decreased the stimulatory effects of diC$_8$ and phospholipase C by 70 and 76% respectively (results not shown). These data suggested that in NIH 3T3 cells the major effects of both PMA and 1,2-DAG on PtdCho synthesis involved protein kinase C.

Comparison of the effects of PMA and bryostatin on PtdCho synthesis and phospholipid hydrolysis

Comparison of the effects of PMA on PtdCho synthesis and phospholipid hydrolysis in rat-1 and NIH 3T3 cells indicated that decreased effects of PMA on phospholipid hydrolysis are not necessarily accompanied by decreased PtdCho synthesis. Next, we examined whether stimulation of phospholipid hydrolysis would automatically lead to increased PtdCho synthesis. For this, a number of protein kinase C activators were compared for their effects on PtdCho synthesis and phospholipid hydrolysis. The most characteristic differences were observed between the effects of PMA and bryostatin, two similarly potent stimulators of protein phosphorylation in intact cells [37], in *A-raf*-transformed NIH 3T3 cells and in HL60 human leukaemia cells. In the untransformed NIH 3T3 cells both PMA (100 nM) and bryostatin (100 nM) stimulated both PtdCho synthesis and phospholipid hydrolysis, with bryostatin being somewhat less effective than PMA (Table 5). In *A-raf*-transformed cells, PMA stimulated the synthesis of PtdCho about 1.7-fold, whereas bryostatin had no stimulatory effect at all. At the same time, bryostatin stimulated, although less effectively than PMA did, the hydrolysis of PtdCho and PtdEtn 3.9- and 3.3-fold respectively (Table 5). In HL60 cells, bryostatin also failed to stimulate PtdCho synthesis, despite its significant (4.1–4.8-fold) stimulatory effects on phospholipid hydrolysis. On the other hand, the stimulatory effects of PMA on PtdCho synthesis and phospholipid hydrolysis in HL60 cells were similar to those observed in *A-raf*-transformed cells (Table 5). From these data it seems clear that stimulation of phospholipase D-mediated hydrolysis of phospholipids, such as observed with bryostatin, does not automatically lead to increased PtdCho synthesis.

To understand better the differences between the effects of PMA and bryostatin, we examined conditions which might enhance the ability of bryostatin to stimulate PtdCho synthesis in

Table 5. Effects of PMA and bryostatin on phospholipid metabolism in untransformed and transformed cells

For the determination of PtdCho synthesis, [^{14}C]choline was used as the labelling agent. The ^{14}C content of PtdCho in untreated NIH 3T3, NIH 3T3/*A-raf* and HL60 cells was 14100 $\pm$ 470, 18310 $\pm$ 670 and 6620 $\pm$ 220 d.p.m./2 h per 10^6 cells respectively. For the determination of phospholipid hydrolysis by phospholipase D, cells were prelabelled with [^{14}C]choline or [^{14}C]ethanolamine for 48 h, followed by incubation of cells for 1 h in the absence or presence of PMA (100 nM) or bryostatin (100 nM). The ^{14}C content of choline released from untreated NIH 3T3, NIH 3T3/*A-raf* and HL60 cells was 1780 $\pm$ 80, 1590 $\pm$ 310 and 2370 $\pm$ 160 d.p.m./h per 10^6 cells respectively. The ^{14}C content of ethanolamine released from untreated cells in the same order was 3180 $\pm$ 440, 3960 $\pm$ 370 and 2620 $\pm$ 190 d.p.m./h per 10^6 respectively. Data are means $\pm$ S.E.M. of three determinations and are expressed as percentages of control (100%). Similar results were obtained in two other experiments.

Cell line	Addition	PtdCho synthesis	PtdCho hydrolysis	PtdEtn hydrolysis
NIH 3T3	None	100 $\pm$ 4.3	100 $\pm$ 18.4	100 $\pm$ 5.5
	PMA	263 $\pm$ 2.2	700 $\pm$ 10.1	356 $\pm$ 3.1
	Bryostatin	203 $\pm$ 2.6	574 $\pm$ 14.5	307 $\pm$ 3.3
NIH 3T3/*A-raf*	None	100 $\pm$ 2.1	100 $\pm$ 14.9	100 $\pm$ 2.6
	PMA	170 $\pm$ 3.0	639 $\pm$ 6.7	499 $\pm$ 3.3
	Bryostatin	104 $\pm$ 2.2	394 $\pm$ 6.2	333 $\pm$ 8.9
HL60	None	100 $\pm$ 1.9	100 $\pm$ 2.9	100 $\pm$ 1.3
	PMA	182 $\pm$ 4.7	618 $\pm$ 4.7	496 $\pm$ 11.6
	Bryostatin	102 $\pm$ 3.3	486 $\pm$ 13.3	411 $\pm$ 7.4

Table 6. Effects of ionomycin, PMA and bryostatin on PtdCho synthesis in untransformed and *A-raf*-transformed NIH 3T3 fibroblasts

Cells were incubated with [^{14}C]choline for 2 h in the absence or presence of PMA (100 nM), bryostatin (100 nM) and/or ionomycin (2 μM). Data are means $\pm$ S.E.M. of four determinations. Similar results were obtained in another experiment.

	Incorporation of [^{14}C]choline into PtdCho (d.p.m./2 h per 10^6 cells)			
	NIH 3T3		NIH 3T3/*A-raf*	
Addition	None	Ionomycin	None	Ionomycin
None	15280 $\pm$ 660	18420 $\pm$ 890	21340 $\pm$ 1180	21720 $\pm$ 1050
PMA	42690 $\pm$ 340	42730 $\pm$ 240	33300 $\pm$ 720	36070 $\pm$ 510
Bryostatin	35120 $\pm$ 390	37540 $\pm$ 810	21540 $\pm$ 450	27420 $\pm$ 410

the transformed cells. As shown in Table 6, addition of the Ca^{2+} ionophore ionomycin to the *A-raf*-transformed cells partially restored the stimulatory effect of bryostatin on PtdCho synthesis. Ionomycin failed to modify the effects of bryostatin or PMA in the untransformed NIH 3T3 cells (Table 6). Importantly, ionomycin (0.1–2 μM) did not modify the stimulatory effects of 100 nM-PMA or 100 nM-bryostatin on the hydrolysis of PtdCho or PtdEtn in either cell line (results not shown). When applied alone, ionomycin slightly (1.2–1.3-fold) stimulated the hydrolysis of both PtdCho and PtdEtn in each cell line studied here.

DISCUSSION

It has been suggested that PMA-stimulated PtdCho synthesis is preceded by, and might possibly be the result of, increased PtdCho hydrolysis [11,23–25]. The following results argue against such direct relationship between phospholipid hydrolysis and PtdCho synthesis. (1) Although PMA was nearly 3 times as effective in NIH 3T3 cells as in rat-1 cells in stimulating phospholipid hydrolysis, it had similar stimulatory effects on PtdCho synthesis in these cell lines. (2) In transformed cells, bryostatin stimulated phospholipid hydrolysis, but it failed to stimulate PtdCho synthesis.

One might still argue that formation of a special 1,2-DAG pool, rather than the extensive hydrolysis of phospholipids, is important for the PMA action on PtdCho synthesis. This is also unlikely, since (1) in the rat-1 cells, stimulation of PtdCho synthesis by PMA was not accompanied by an increase in 1,2-DAG, and (2) the 1,2-DAG-elevating agents, such as phospholipase C or diC_8, were less effective than PMA in stimulating PtdCho synthesis. The reason for this latter phenomenon may be that the 1,2-DAG-elevating agents are less potent than PMA in stimulating protein phosphorylation in intact cells [36–38], and in fibroblasts the main action of 1,2-DAG on PtdCho synthesis appears to be mediated by protein kinase C.

One reason why the action of PMA on PtdCho synthesis remains elusive might be that PMA acts through two different, but co-operatively interacting, mechanisms. Although this remains to be proven, the differential effects of H7 at low and high PMA concentrations and the divergent effects of PMA and bryostatin in the transformed cells are consistent with such a possibility.

Two possible mechanisms may account for the observed differential effects of H7: (1) the effect of PMA on PtdCho synthesis may involve two different isoforms of protein kinase C (regulating two different mechanisms), only one of them being inhibited by H7, or (2) two sub-populations of the same protein kinase C isoform may mediate the effects of PMA. Some regulatory conditions, such as high ATP concentration (which competes with H7 at the substrate-binding site of protein kinase C; [39]), may selectively render one sub-population of protein kinase C insensitive to H7. It appears that in the transformed cells bryostatin cannot interact with an isoform or a sub-population of protein kinase C unless the intracellular concentration of Ca^{2+} is elevated. This points to the possibility that Ca^{2+} is involved in the regulation of PtdCho synthesis.

In summary, our work indicates that there is no direct relationship between PMA-induced phospholipid hydrolysis and PtdCho synthesis. In addition, the results also suggest that the action of PMA may involve two regulatory mechanisms, only one of which is regulated by bryostatin in the transformed cells. These observations may be important in view of the known differential effects of TPA and bryostatin on tumour promotion in mouse skin [44,45], HL60 cell differentiation [46] and T-lymphocyte proliferation [47].

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REFERENCES

1. Nishizuka, Y. (1986) Science **233**, 305–312
2. Paddon, H. B. & Vance, D. E. (1980) Biochim. Biophys. Acta **620**, 636–640
3. Pelech, S. L., Paddon, H. B. & Vance, D. E. (1984) Biochim. Biophys. Acta **795**, 447–451
4. Kolesnick, R. N. (1987) J. Biol. Chem. **262**, 14525–14530
5. Watkins, J. D. & Kent, C. (1990) J. Biol. Chem. **265**, 2190–2197
6. Weinhold, P. A., Feldman, D. A., Quade, M. M., Miller, J. C. & Brooks, R. L. (1981) Biochim. Biophys. Acta **665**, 134–144
7. Pritchard, P. H. & Vance, D. E. (1981) Biochem. J. **196**, 261–267
8. Zelinski, T. A., Savard, J. D., Man, R. Y. K. & Choy, P. C. (1980) J. Biol. Chem. **255**, 11423–11428
9. Post, M., Batenburg, E., Schuurmans, A. J. M. & Van Golde, L. M. G. (1982) Biochim. Biophys. Acta **712**, 390–394
10. Pelech, S. L., Cook, H. W., Paddon, H. B. & Vance, D. E. (1984) Biochim. Biophys. Acta **795**, 433–440
11. Cook, H. W. & Vance, D. E. (1985) Can. J. Biochem. Cell Biol. **63**, 145–151
12. Radika, K. & Possmayer, F. (1985) Biochem. J. **232**, 833–840
13. Exton, J. H. (1990) J. Biol. Chem. **265**, 1–4
14. Kiss, Z. & Anderson, W. B. (1989) J. Biol. Chem. **264**, 1483–1487
15. Kiss, Z. & Anderson, W. B. (1990) J. Biol. Chem. **265**, 7345–7350
16. Cornell, R. & Vance, D. E. (1987) Biochim. Biophys. Acta **919**, 26–36
17. Rosenberg, I. L., Smart, D. A., Gilfillan, A. M. & Rooney, S. A. (1987) Biochim. Biophys. Acta **921**, 473–480
18. Sleight, R. & Kent, C. (1980) J. Biol. Chem. **255**, 10644–10650
19. Sleight, R. & Kent, C. (1983) J. Biol. Chem. **258**, 831–835
20. Tercè, F., Record, M., Chap, H. & Douste-Blazy, L. (1984) Biochem. Biophys. Res. Commun. **125**, 413–419
21. Tercè, F., Record, M., Ribbes, G., Chap, H. & Douste-Blazy, L. (1988) J. Biol. Chem. **263**, 3142–3145
22. Morand, J. N. & Kent, C. (1989) J. Biol. Chem. **264**, 13785–13792
23. Guy, G. R. & Murray, A. W. (1982) Cancer Res. **42**, 1980–1985
24. Grove, R. I. & Schimmel, S. D. (1982) Biochim. Biophys. Acta **711**, 272–280
25. Daniel, L. W., Waite, M. & Wykle, R. L. (1986) J. Biol. Chem. **261**, 9128–9132
26. Pettit, G. R., Herald, C. L., Doubek, D. L. & Herald, D. L. (1982) J. Am. Chem. Soc. **104**, 6846–6848
27. Kobayashi, M. & Kanfer, J. N. (1987) J. Neurochem. **48**, 1597–1603
28. Beck, T. W., Huleihel, M., Gunnel, M., Bonner, T. I. & Rapp, U. R. (1987) Nucleic Acids Res. **15**, 595–609
29. Collins, S. J., Gallo, R. C. & Gallagher, R. E. (1977) Nature (London) **270**, 347–349
30. Cook, S. J. & Wakelam, M. J. O. (1989) Biochem. J. **263**, 581–587
31. Kiss, Z. & Farkas, T. (1975) Biochem. Pharmacol. **24**, 999–1002

32. Kiss, Z. (1976) Eur. J. Biochem. **67**, 557–561
33. Liscovitch, M. (1989) J. Biol. Chem. **264**, 1450–1456
34. Preiss, J. E., Loomis, C. R., Bell, R. M. & Niedel, J. E. (1986) Methods Enzymol. **141**, 194–200
35. Billah, M. M. & Anthes, J. C. (1990) Biochem. J. **269**, 281–291
36. Kiss, Z. & Steinberg, R. A. (1985) Cancer Res. **45**, 2732–2740
37. Kiss, Z., Deli, E., Shoji, M., Koeffler, H. P., Pettit, G. R., Vogler, W. R. & Kuo, J. F. (1987) Cancer Res. **47**, 1302–1307
38. Kiss, Z., Deli, E. & Kuo, J. F. (1987) FEBS Lett. **213**, 365–371
39. Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984) Biochemistry **23**, 5036–5041
40. Rodriguez-Pena, A. & Rozengurt, E. (1984) Biochem. Biophys. Res. Commun. **120**, 1053–1059
41. Blackshear, P. J., Witters, L. A., Girard, P. R., Kuo, J. F. & Quamo, S. N. (1985) J. Biol. Chem. **260**, 13304–13315
42. Hovis, J. G., Stumpo, D. J., Halsey, D. L. & Blackshear, P. J. (1986) J. Biol. Chem. **261**, 10380–10386
43. Kolesnick, R. N. & Paley, A. E. (1987) J. Biol. Chem. **262**, 9204–9210
44. Hennings, H., Blumberg, P. M., Pettit, G. R., Herald, C. L., Shores, R. & Yuspa, S. H. (1987) Carcinogenesis **8**, 1343–1346
45. Sako, T., Yuspa, S. H., Herald, C. L., Pettit, G. R. & Blumberg, P. M. (1987) Cancer Res. **47**, 5445–5450
46. Kraft, A. S., William, F., Pettit, G. R. & Lilly, M. B. (1989) Cancer Res. **49**, 1287–1293
47. Hess, A. D., Silanskis, M. K., Esa, A. H., Pettit, G. R. & May, W. S. (1988) J. Immunol. **141**, 3263–3269

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