Antagonism of phorbol-ester-stimulated phosphatidylcholine biosynthesis by the phospholipid analogue hexadecylphosphocholine

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The antagonization of phorbol 12-myristate 13-acetate (PMA)-stimulated phosphatidylcholine (PtdCho) biosynthesis by the phospholipid analogue hexadecylphosphocholine (HePC) in MDCK cells was investigated and compared with the corresponding influence in HeLa cells. In both cell lines, PMA-stimulated PtdCho biosynthesis was antagonized by 50 μ M HePC. However, subsequent experiments provided evidence that PMA enhances PtdCho biosynthesis by at least two mechanisms: (i) by stimulation of choline uptake and (ii) by translocation of CTP:choline phosphate cytidylyltransferase to membranes. In MDCK cells, 5 nM PMA caused a 4-fold increase in [*methyl*- 3 H]choline incorporation into PtdCho, which was paralleled by an approx. 2-fold stimulation of choline uptake. These data indicate that choline uptake might play an important role in the

regulation of PtdCho biosynthesis in this cell line, especially since we could not detect any significant increase in membrane-bound cytidylyltransferase activity in PMA-treated MDCK cells. In contrast, enhanced PtdCho biosynthesis in HeLa cells is achieved by a 2-fold increase in particulate cytidylyltransferase activity after PMA stimulation. Translocation of cytidylyltransferase from the cytosol to membranes is therefore important in HeLa cells. Nevertheless, in both cell lines, the main target of HePC seems to be the translocation process. In MDCK cells, addition of 50 μ M HePC decreases membrane-bound cytidylyltransferase activity by about 45 %, compared with control cells and PMA-treated cells. In HeLa cells, PMA-induced translocation of cytidylyltransferase to membranes is totally abolished by HePC.

INTRODUCTION

The CDP-choline pathway is by far the most important route of phosphatidylcholine (PtdCho) biosynthesis in different cell types. CTP:choline phosphate cytidylyltransferase (EC 2.7.7.15) is the rate-limiting enzyme of this pathway (Pelech and Vance, 1984; Tjibburg et al., 1989; Vance, 1990; Kent et al., 1991), and its activity seems to be regulated by movement between membranes (active form) and cytosol (inactive form), especially in HeLa cells (Vance and Pelech, 1984). This translocation process is modulated by the presence or absence of fatty acids in the culture medium of cells (Pelech et al., 1984a; Aeberhard et al., 1986; Cornell and Vance, 1987; Weinhold et al., 1991). However, the relevance of a direct regulation of cytidylyltransferase by fatty acids in animals is unknown. More recently, two other mechanisms of cytidylyltransferase regulation were reported: (i) feedback regulation by the cellular PtdCho content (Jamil et al., 1990; Yao et al., 1990), and (ii) cytidylyltransferase activation by an increased *sn*-1,2-diacylglycerol content of cells (Kolesnick and Hermer, 1990).

Phorbol esters, e.g. phorbol 12-myristate 13-acetate (PMA), activate PtdCho biosynthesis in different cell types (Paddon and Vance, 1980; Guy and Murray, 1982; Cook et al., 1989). The mechanism of phorbol-ester-stimulated PtdCho biosynthesis has been the subject of many studies. Vance and co-workers showed that PMA causes the translocation of cytidylyltransferase from cytosol to membranes in HeLa cells (Cook and Vance, 1984; Pelech et al., 1984b) and suggested that the translocation is mediated by an increased cellular diacylglycerol content (Utal et al., 1991). Kent and co-workers confirmed the effect of PMA on PtdCho biosynthesis, but did not detect a translocation of cytidylyltransferase (Watkins and Kent, 1990). However, both

groups agreed that PMA has no effect on the phosphorylation of cytidylyltransferase.

Recently, the alkylphosphocholine, hexadecylphosphocholine (HePC), was shown to decrease PtdCho biosynthesis in Madin–Darby canine kidney (MDCK) cells (Haase et al., 1991) by inhibiting the translocation of cytidylyltransferase to membranes (Geilen et al., 1992). In the present study, HePC was used as a tool for investigating phorbol-ester-stimulated PtdCho biosynthesis. It was shown that HePC antagonizes phorbol-ester-stimulated PtdCho biosynthesis in MDCK and HeLa cells. By comparison of the effects of PMA and HePC on both cell lines, our data provide evidence that phorbol ester stimulation of PtdCho biosynthesis occurs by different mechanisms. In agreement with Vance and co-workers, we showed that the phorbol ester stimulation of PtdCho biosynthesis in HeLa cells is caused by cytidylyltransferase translocation to membranes, and that HePC antagonizes this translocation process. PMA also stimulated PtdCho biosynthesis in MDCK cells, but an involvement of cytidylyltransferase translocation was not detectable. Additional treatment of the cells with HePC caused a decrease in phorbol-ester-stimulated PtdCho biosynthesis below the control level. Moreover, this inhibition of PtdCho biosynthesis was accompanied by a decrease in membrane-bound cytidylyltransferase in MDCK cells which had been treated with PMA plus HePC. These data indicate that HePC impairs cytidylyltransferase translocation in MDCK cells, irrespective of whether PMA is present in the medium or not.

EXPERIMENTAL

Materials

[*methyl*- 3 H]Choline chloride (2.8–3.1 TBq/mmol), [*methyl*- 14 C]-

Abbreviations used: BCA, bicinechonic acid; MDCK, Madin–Darby canine kidney; Me₂SO, dimethyl sulphoxide; HePC, hexadecylphosphocholine; PMA, phorbol 12-myristate 13-acetate; PtdCho, phosphatidylcholine.

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phosphocholine (2.04 GBq/mmol) and [γ - 32 P]ATP (~ 185 TBq/mmol) were from Amersham (Braunschweig, Germany). HePC was synthesized by the method of Knopik et al. (1991). Silica-gel 60 high-performance t.l.c. plates and all solvents and reagents (reagent grade) were from E. Merck (Darmstadt, Germany). The bicinchoninic acid (BCA) kit for protein determination was from Pierce (Weiskirchen, Germany). Dithiothreitol, phenylmethanesulphonylfluoride, miscellaneous lipids, *sn*-1,2-diacylglycerol, PMA, digitonin and PtdCho precursors were from Sigma (München, Germany). *sn*-1,2-Diacylglycerol kinase from *Escherichia coli* was from Lipidex Inc. (Westfield, NJ, U.S.A.). Collagen type I was prepared from rat tails. A Berthold LB 2821 HR t.l.c. scanner (Berthold, Wildbad, Germany) was used for quantification of radioactivity, and a video densitometer (Fischer Biotech, Reiskirchen, Germany) was used for lipid quantification.

Cell culture and labelling of cells

MDCK and HeLa cells were given by Dr. R. T. C. Huang, of this Institute. They were grown in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal-calf serum, 0.56 g/l glutamine, 100 000 i.u./l penicillin and 0.1 g/l streptomycin in plastic culture dishes (Nunc, Wiesbaden, Germany). Media and culture reagents were from Gibco (Karlsruhe, Germany). Penicillin and streptomycin were from Boehringer (Mannheim, Germany). Cells were subcultured by using 0.1% trypsin in PBS supplemented with 0.02% EDTA. For experimental purposes cells were used on day 3 of culture. After 3 h of preincubation, [*methyl*- 3 H]choline labelling was initiated by adding the appropriate pulse medium. The different pulse media (2.5 μ Ci/ml) and the media used for the preincubation contained 5 nM PMA or 5 nM PMA plus 50 μ M HePC for MDCK cells, and 100 nM PMA or 100 nM PMA plus 50 μ M HePC for HeLa cells; control media contained 0.005% dimethyl sulphoxide (Me_2SO) for MDCK cells and 0.1% Me_2SO for HeLa cells.

For pulse-chase experiments, MDCK cells were labelled with [*methyl*- 3 H]choline (2.5 μ Ci/ml) for 2 h. Pulse medium was removed, the cells were washed three times with PBS, and chase medium was added containing unlabelled choline (0.58 mM). Additionally, the chase media contained 5 nM PMA or 5 nM PMA plus 50 μ M HePC; control media contained 0.005% Me_2SO .

Extraction, separation and quantification of lipids and water-soluble metabolites

After incubation with pulse medium, cells were washed twice with ice-cold PBS (pH 7.2) and harvested with a cell lifter (Costar; Cambridge, CA, U.S.A.), followed by modified lipid extraction by the method of Bligh and Dyer (1959) as described previously (Geilen et al., 1992). Lipids were separated as described by Skipski et al. (1964), with the solvent chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). Phosphatidylcholine precursors were separated in methanol/0.6% NaCl/aq. 25% NH_3 (8:5:1, by vol.). Radioactivity was quantified by radioscanning. For lipid quantification, all lipids were stained with a CuSO_4 solution (156 g/l in 8.5% H_3PO_4) by the method of Touchstone et al. (1980) and quantified by video densitometry. Staining was linear in the range 0.5–6 μ g of lipid per band.

Choline uptake

MDCK cells were grown to confluence on plastic foils (35 mm diameter) which had been previously sterilized with 70% (v/v)

ethanol and subsequently coated with collagen type I. The cells were preincubated for 4 h with medium containing PMA or PMA plus HePC in the same concentrations as used in the labelling experiments. Then uptake was determined as described elsewhere (Geilen et al., 1992).

Digitonin-mediated release of cytidyltransferase from MDCK and HeLa cells

The release of cytidyltransferase from MDCK and HeLa cells was measured as described by Pelech et al. (1984a). Cells were incubated for 4 h in 35 mm dishes with medium containing the appropriate supplements (as described for the labelling experiments), then washed with 2×1 ml of ice-cold PBS. Digitonin-mediated release of cytidyltransferase was performed as described by Geilen et al. (1992). From the data, the distribution of cytidyltransferase activity between cytosol and membranes was calculated for each dish.

CTP:phosphocholine cytidyltransferase assay

The enzyme activity was measured by a modification of the method of Sohal and Cornell (1990) as described previously (Geilen et al., 1992), by using liposomes (400 μ M PtdCho and 400 μ M oleic acid) and 10 μ l of enzyme preparation in a final volume of 55 μ l. One unit of enzyme activity is defined as 1 nmol of CDP-choline formed/min.

sn-1,2-Diacylglycerol determination in MDCK cells

The *sn*-1,2-diacylglycerol assay was carried out as described by Preiss et al. (1986). Briefly, PMA-treated, PMA + HePC-treated and control cells were extracted by the method of Bligh and Dyer (1959) as described above. A sample of the chloroform phase was evaporated and solubilized in 20 μ l of a detergent solution (7.5% octyl β -D-glucoside and 5 mM cardiolipin in 1 mM diethylenetriaminepenta-acetic acid) by sonication. Then the *sn*-1,2-diacylglycerol content of the samples was determined as described by Geilen et al. (1992).

Other procedures

Cellular protein was determined in a sample from each radiolabelling experiment by the BCA assay (Smith et al., 1985), with BSA as a standard.

Cytotoxicity of HePC to HeLa cells was measured by alkaline phosphatase release as described by Culvenor et al. (1981).

Proliferation of HeLa cells was measured by determination of the cell number by the method of Gillies et al. (1986).

Statistical comparisons were made in these studies by Student's *t* test.

RESULTS

Phorbol ester stimulation of phosphatidylcholine biosynthesis in MDCK cells is antagonized by HePC

In a recent study we showed that PtdCho biosynthesis is stimulated by PMA treatment (5 nM) in MDCK cells (Geilen et al., 1992). To confirm this finding, MDCK cells, which had been grown to confluence, were incubated with pulse medium containing 2.5 μ Ci of [*methyl*- 3 H]choline/dish in the absence or the presence of 5 nM PMA. The time-dependent incorporation of [*methyl*- 3 H]choline into choline, phosphocholine and PtdCho of MDCK cells under these conditions is shown in Figure 1. PMA caused a 4-fold increase of label in PtdCho after 6 h of incubation

Figure 1 Effect of PMA and PMA + HePC on PtdCho biosynthesis in MDCK cells

MDCK cells grown to confluence were preincubated with 5 nM PMA, or 5 nM PMA plus 50 μ M HePC for 3 h. Controls contained 0.005% Me₂SO. Pulse medium was added containing 7.4×10^4 Bq of [*methyl*-³H]choline/ml and 5 nM PMA (○) or 5 nM PMA plus 50 μ M HePC (□). For control experiments 0.005% Me₂SO was added (●). After different incubation times as indicated in the Figure, the cells were mechanically harvested and PtdCho and PtdCho precursors were analysed as described in the Experimental section. The values of incorporated radioactivity are given in d.p.m./mg of cellular protein (means $\pm$ S.D., $n = 3$).

Table 1 Effect of PMA and PMA + HePC on the lipid composition of MDCK cells

MDCK cells grown to confluence were incubated with 5 nM PMA, or 5 nM PMA + 50 μ M HePC, or with 0.005% Me₂SO as a control. After 7 h, the cells were mechanically harvested and the lipid composition was analysed as described in the Experimental section. The values are given as percentages of total lipids (means $\pm$ S.D., $n = 3$); * significantly different from controls at $P < 0.05$; ** significantly different from controls at $P < 0.02$. Abbreviations: SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; CL, cardiolipin; TG, triacylglycerols.

Treatment of cells	Composition (%)							Total lipids (μ g/ 10^6 cells)
	SM	PtdCho	PS	PI	PE	CL	TG	
Control	6.1 $\pm$ 1.0	34.7 $\pm$ 0.5	5.6 $\pm$ 1.0	6.7 $\pm$ 1.2	21.1 $\pm$ 1.1	13.3 $\pm$ 1.9	12.5 $\pm$ 1.1	90.1 $\pm$ 15.2
PMA	6.7 $\pm$ 0.9	37.8 $\pm$ 1.4*	3.2 $\pm$ 0.2**	4.9 $\pm$ 0.5	20.1 $\pm$ 0.6	13.2 $\pm$ 0.3	13.1 $\pm$ 0.1	91.3 $\pm$ 2.0
PMA + HePC	6.3 $\pm$ 1.8	35.0 $\pm$ 1.2	4.9 $\pm$ 1.1	6.2 $\pm$ 0.4	23.5 $\pm$ 1.0	12.8 $\pm$ 2.0	11.4 $\pm$ 0.9	96.0 $\pm$ 7.2

with pulse medium as compared with control cells. The specific radioactivity of labelled PtdCho increased from 792 ± 6 d.p.m./ μ g of PtdCho in control cells to 3072 ± 640 d.p.m./ μ g in PMA-treated cells after 6 h of pulse. Simultaneously, PMA treatment enhanced the radiolabelling of the choline pool and decreased the radiolabelling of the phosphocholine pool. These results indicate that PMA treatment may influence the choline uptake and the turnover of the aqueous precursors of PtdCho in MDCK cells. Alterations of the radioabelling of the CDP-choline pool were not detectable in these experiments, because this pool represented less than 1% of the total aqueous metabolites.

HePC was shown to antagonize PMA-induced morphological alterations of MDCK cells (Geilen et al., 1991). We therefore investigated the effect of HePC on PMA-stimulated PtdCho biosynthesis in MDCK cells. Figure 1 shows that the PMA-induced effect on the aqueous precursors and on PtdCho biosynthesis was totally abolished by treatment of MDCK cells with 5 nM PMA plus 50 μ M HePC. The specific radioactivity of PtdCho in PMA + HePC-treated cells was 416 ± 40 d.p.m./ μ g of PtdCho after 6 h of pulse. This value is 47% lower than the control experiment.

The lipid composition of MDCK cells was also altered by PMA treatment (Table 1). Whereas the relative contents of sphingomyelin, phosphatidylethanolamine, cardiolipin and triacylglycerols seemed to be unaffected, the relative content of PtdCho was increased in PMA-treated cells. The relative contents of phosphatidylserine and phosphatidylinositol were slightly decreased. Treatment of MDCK cells with 5 nM PMA plus 50 μ M HePC resulted in a lipid composition comparable with that of control cells.

Phorbol esters do not cause any detectable translocation of cytidylyltransferase in MDCK cells

In order to examine the mechanism of PMA stimulation in MDCK cells, the digitonin-mediated release of cytosolic cytidylyltransferase was measured. The data presented in Table 2 do not show a significant translocation of cytidylyltransferase activity from cytosol to membranes in cells treated with PMA, although we determined cytidylyltransferase activity in the released cytosolic as well as in the particulate fraction. After 1 min of digitonin treatment, 55% (0.63 nmol/min per 10^6 cells) of the total cytidylyltransferase activity was released from control cells,

Table 2 Effect of PMA and PMA + HePC on the distribution of cytidylyltransferase in MDCK cells

The translocation of cytidylyltransferase in MDCK cells was measured by digitonin-mediated release of cytosolic cytidylyltransferase. Cells were incubated for 4 h in 35 mm dishes with medium containing 5 nM PMA, or 5 nM PMA + 50 μ M HePC. Controls contained 0.005% Me₂SO. After incubation, cytidylyltransferase activity was measured in the digitonin supernatants and in the cell ghost homogenates as described in the Experimental section. From the data, the distribution (%) of cytosolic and membrane-bound (Membr.) cytidylyltransferase activity was calculated for each dish ($n = 4$; * significantly different from controls at $P < 0.05$; ** significantly different from controls at $P < 0.02$; *** significantly different from controls at $P < 0.01$). For 1 min of digitonin release, the total activity was 1.15 ± 0.15 nmol/ 10^6 cells in controls, 1.34 ± 0.12 in PMA-treated and 1.29 ± 0.15 in PMA + HePC-treated MDCK cells. The experiment was repeated, and similar results were obtained.

Digitonin treatment	Distribution (%)					
	Control		PMA		PMA + HePC	
	Cytosol	Membr.	Cytosol	Membr.	Cytosol	Membr.
1 min	54.8 $\pm$ 0.5	45.2 $\pm$ 0.5	47.7 $\pm$ 2.1	52.3 $\pm$ 2.1**	75.1 $\pm$ 6.6	24.9 $\pm$ 6.6***
3 min	65.5 $\pm$ 2.8	34.5 $\pm$ 2.8	58.1 $\pm$ 5.9	41.9 $\pm$ 5.9	74.9 $\pm$ 4.9	25.1 $\pm$ 4.9*
6 min	74.1 $\pm$ 1.6	25.9 $\pm$ 1.6	77.7 $\pm$ 1.5	22.3 $\pm$ 1.5	87.8 $\pm$ 3.1	12.2 $\pm$ 3.1***

Figure 2 Influence of PMA and PMA + HePC on the [methyl-³H]choline incorporation into PtdCho of MDCK cells

MDCK cells were labelled with [methyl-³H]choline (2.5 μ Ci/ml) for 2 h. Pulse medium was removed, the cells were washed three times with PBS and chase medium was added containing unlabelled choline (0.58 mM) and 5 nM PMA (○) or 5 nM PMA plus 50 μ M HePC (□); control media contained 0.005% Me₂SO (●). At different time points, the cells were extracted by the method of Bligh and Dyer (1959) and the lipids were determined as described in the Experimental section. Each point represents the mean of two independent experiments.

whereas in PMA-treated cells 48% (0.64 nmol/min per 10^6 cells) was released. The corresponding particulate cytidylyltransferase activities were 45% (0.52 nmol/min per 10^6 cells) for controls and 52% (0.71 nmol/min per 10^6 cells) for PMA-treated cells. These data actually show a slight increase in particulate cytidylyltransferase activity after treatment with phorbol esters. On the other hand, when the analysis was performed after 6 min of digitonin permeabilization, 26% (0.32 nmol/min per 10^6 cells) of total cytidylyltransferase activity was found in the membrane fraction of control cells and only 22% (0.28 nmol/min per 10^6 cells) in the membrane fraction of cells treated with PMA. Taken together, these data demonstrate that there is no detectable translocation of cytidylyltransferase in PMA-treated MDCK cells that could be responsible for the 4-fold increase in PtdCho biosynthesis. To prove this finding, pulse-chase experiments were carried out. MDCK cells were labelled with [methyl-³H]choline for 2 h. Then pulse medium was removed and chase medium was added containing 5 nM PMA or 5 nM PMA plus 50 μ M HePC respectively. Figure 2 shows that the choline

incorporation into PtdCho is not altered in PMA-treated cells as compared with controls. HePC decreases the incorporation rate of [methyl-³H]choline into PtdCho in the presence of PMA.

The situation was somewhat different when cells were incubated with PMA + HePC. After 1 min of digitonin treatment, 75% (0.98 nmol/min per 10^6 cells) of total cytidylyltransferase activity resides in the cytosol, and 25% (0.32 nmol/min per 10^6 cells) in the particulate fraction. This increased release of cytosolic cytidylyltransferase can be measured at three different time points of digitonin permeabilization. Furthermore, these data coincide with previously published data dealing with the influence of HePC on cytidylyltransferase translocation in MDCK cells (Geilen et al., 1992). Hence, it seems reasonable that HePC inhibits enhanced PtdCho biosynthesis by the mechanism that we have described for non-stimulated cells (Geilen et al., 1992).

Diacylglycerol content of MDCK cells is not affected by phorbol ester treatment

The effect of PMA on the cellular *sn*-1,2-diacylglycerol content was investigated in MDCK cells and HeLa cells. In HeLa cells, PMA treatment leads to a 1.6-fold increase of diacylglycerol after 2 h as compared with controls, which is in agreement with previously published data (Utal et al., 1991). To determine the time-dependent effect of PMA treatment on the diacylglycerol level of MDCK cells, the cells were incubated with medium containing PMA. The amount of diacylglycerol was measured at different time points (Table 3). Compared with control experiments, no difference was found up to 6 h. In experiments, in which cells were incubated with medium containing PMA plus HePC, an increase in the diacylglycerol content was seen. This increase agrees with data obtained previously from the HePC treatment of MDCK cells without PMA stimulation (Geilen et al., 1992).

Phorbol-ester-enhanced choline uptake is antagonized by HePC in MDCK cells

During the pulse-labelling experiments, there was an approx. 2.5-fold increase in the total amount of [methyl-³H]choline incorporated into phorbol-ester-treated MDCK cells. These findings suggested that PMA influences choline uptake. To verify this assumption, the choline uptake of PMA- and HePC-treated cells was measured under the same conditions as described for experiments on PtdCho biosynthesis in the present study. After

Table 3 Influence of PMA and PMA + HePC on the cellular *sn*-1,2-diacylglycerol content of MDCK cells and HeLa cells

MDCK cells were incubated for up to 6 h with 5 nM PMA, or 5 nM PMA plus 50 μ M HePC, or with 0.005% Me₂SO as a control, and then extracted by the method of Bligh and Dyer (1959). The *sn*-1,2-diacylglycerol (DAG) content was determined as described in the Experimental section. More than 90% of the radioactivity co-chromatographed with phosphatidic acid. The amount of DAG present in the samples was calculated from the sample volume and a DAG standard curve ($n = 3$; *significantly different from controls at $P < 0.01$; **significantly different from controls at $P < 0.001$).

Cells	Incubation time	DAG content (nmol/10 ⁶ cells)		
		Control	PMA	PMA + HePC
MDCK	0.0 h	5.3 $\pm$ 1.0	5.3 $\pm$ 1.0	5.3 $\pm$ 1.0
	0.5 h	7.6 $\pm$ 1.0	6.2 $\pm$ 1.3	7.3 $\pm$ 0.3
	2.0 h	6.3 $\pm$ 1.2	6.1 $\pm$ 0.2	7.4 $\pm$ 0.9
	6.0 h	4.8 $\pm$ 0.9	4.4 $\pm$ 1.1	6.2 $\pm$ 1.2
HeLa	0.0 h	2.3 $\pm$ 0.6	2.3 $\pm$ 0.6	2.3 $\pm$ 0.6
	2.0 h	3.2 $\pm$ 0.6	4.9 $\pm$ 0.7*	6.9 $\pm$ 0.5**

Figure 3 Choline uptake of normal, PMA and PMA + HePC-treated MDCK cells

MDCK cells were grown to confluence on collagen-coated plastic foils (35 mm diam.) and preincubated for 4 h with medium containing 5 nM PMA (○), or 5 nM PMA plus 50 μ M HePC (□), or with 0.005% Me₂SO as a control (●). Choline uptake was determined at 37 °C as described in the Experimental section. Uptake was calculated as the difference between total radioactivity and contaminating radioactivity. Values of uptake are given in pmol/10⁶ MDCK cells (means $\pm$ S.D., $n = 3$; *significantly different from controls at $P < 0.02$).

preincubation with the appropriate media for 4 h (when stimulation of [*methyl*-³H]choline incorporation into PtdCho occurs), the uptake was determined for up to 8 min (Figure 3). In medium containing 28 μ M choline the uptake by 10⁶ MDCK cells was 47 pmol of choline/min for controls and 80 pmol of choline/min for PMA-treated cells. This increase in choline uptake was abolished when PMA+HePC was added to the medium. Although the enhanced uptake does not precisely correspond to the 4-fold stimulation of PtdCho biosynthesis, the results indicate that choline uptake may be a target of phorbol esters in MDCK cells.

Evidence for different mechanisms of phorbol ester action in MDCK and HeLa cells

There is disagreement in the literature concerning the mechanism of PMA stimulation of PtdCho biosynthesis. We therefore

Figure 4 Effect of PMA and PMA + HePC on PtdCho biosynthesis of HeLa cells

HeLa cells grown to confluence were preincubated with 100 nM PMA, or 100 nM PMA plus 50 μ M HePC, or with 0.1% Me₂SO as a control for 3 h. Pulse medium was added containing 7.4×10^4 Bq of [*methyl*-³H]choline/ml and 100 nM PMA (○), or 100 nM PMA plus 50 μ M HePC (□). For control experiments 0.1% Me₂SO was added (●). After different incubation times as indicated in the Figure, the cells were mechanically harvested and PtdCho was analysed as described in the Experimental section. The values of incorporated radioactivity are given in d.p.m./mg of cellular protein (means $\pm$ S.D., $n = 3$).

repeated the experiments on cytidyltransferase translocation, using HeLa cells. This well-characterized cell line is phorbol-ester-sensitive, and stimulation of PtdCho biosynthesis occurs via activation of cytidyltransferase by translocation of the inactive cytosolic form to membranes, as recently demonstrated (Utal et al., 1991).

First of all, we determined the influence of HePC on cell proliferation and cell viability of HeLa cells. HePC inhibits cell proliferation of HeLa cells at concentrations higher than 10 μ M, whereas cell viability is hardly affected up to 100 μ M HePC (viability > 80%; results not shown). Therefore, the subsequent experiments with HeLa cells were performed by incubating confluent cells with 100 nM PMA as described previously (Utal et al., 1991) and with 50 μ M HePC.

As shown in Figure 4, 100 nM PMA caused a 3-fold stimulation of PtdCho biosynthesis, compared with controls. In contrast with MDCK cells, this increase in PtdCho biosynthesis is not accompanied by an enhanced incorporation of total choline label, since the decrease in radiolabel in the huge phosphocholine pool of treated cells corresponds to the increase in radiolabel in the PtdCho pool (results not shown). Nevertheless, phorbol-ester-stimulated PtdCho biosynthesis is also antagonized by HePC in HeLa cells.

In a second set of experiments we measured cytidyltransferase translocation in HeLa cells, using the same procedure as described for MDCK cells. After 1 min of digitonin treatment, there was an approx. 2-fold increase in membrane-bound cytidyltransferase activity in PMA-treated cells, compared with controls (Table 4); and the time-dependent release of cytosolic cytidyltransferase activity in PMA-treated cells was slower than in control cells (results not shown). Moreover, simultaneous addition of HePC to the culture medium totally abolished PMA-stimulated translocation of cytidyltransferase to membranes (Table 4). Although there are some differences in total enzyme activities and in the time course of digitonin permeabilization, we agree with Utal et al. (1991) that stimulation of PtdCho

Table 4 Effect of PMA and PMA + HePC on the distribution of cytidylyltransferase in HeLa cells

The translocation of cytidylyltransferase in HeLa cells was measured by digitonin-mediated release of cytosolic cytidylyltransferase. Cells were incubated for 4 h in 35 mm dishes with medium containing 100 nM PMA, or 100 nM PMA + 50 μ M HePC, or 0.1 % Me₂SO as a control. After incubation, cytidylyltransferase activity was measured in the digitonin supernatants released after 1 min and in the cell ghost homogenates as described in the experimental section. From the data, the distribution (%) of cytosolic and membrane-bound (Membr.) cytidylyltransferase activity was calculated for each dish ($n = 3$; * significantly different from controls at $P < 0.01$). For the 1 min time point, the total activity was 1.26 nmol/min per 10^6 cells in PMA-treated cells, 1.50 in PMA + HePC-treated cells and 1.40 in controls.

Treatment	Distribution (%)	
	Cytosol	Membr.
Control	75.4 $\pm$ 3.1	24.6 $\pm$ 3.1
PMA	48.1 $\pm$ 2.2	51.9 $\pm$ 2.2*
PMA + HePC	76.5 $\pm$ 0.9	23.5 $\pm$ 0.9

biosynthesis in HeLa cells is achieved by an enhanced translocation of cytidylyltransferase, which is mediated by an increased diacylglycerol content (Table 3).

DISCUSSION

We have shown in this study that phorbol ester treatment of MDCK cells increased the specific labelling of PtdCho, indicating that PtdCho biosynthesis is stimulated. This stimulation was antagonized by HePC. In order to examine the underlying mechanism, the translocation of cytidylyltransferase was determined in PMA- and PMA + HePC-treated MDCK cells. Unexpectedly, no differences in the cytidylyltransferase distribution in PMA-treated and control cells were detectable. These results coincided with data showing that the cellular diacylglycerol content of MDCK cells is not affected by PMA treatment and that in pulse-chase studies incorporation of choline into PtdCho is not increased. However, simultaneous treatment of MDCK cells with HePC + PMA led to an enhanced release of cytosolic cytidylyltransferase, indicating that the translocation process is also impaired by HePC in PMA-stimulated cells.

To evaluate the data obtained on cytidylyltransferase translocation in PMA-treated MDCK cells, we decided to repeat this experiment with HeLa cells. In this cell line, an enhancement of cytidylyltransferase translocation to membranes by PMA treatment has been reported (Utal et al., 1991); it should therefore be a useful positive control. Indeed, we showed that PMA treatment of HeLa cells stimulated PtdCho biosynthesis by enhancing the translocation of cytidylyltransferase to membranes. Both effects were antagonized by additional HePC treatment. Moreover, an increase of the cellular diacylglycerol content was detectable in PMA-treated HeLa cells, which is in agreement with data of Utal et al. (1991). It is interesting that simultaneous addition of HePC further elevated the diacylglycerol content of stimulated HeLa cells. This is consistent with the statement that impaired translocation of cytidylyltransferase by HePC is not mediated via a decrease in cellular diacylglycerol.

In contrast with HeLa cells, regulation of choline uptake in MDCK cells may influence the rate of PtdCho biosynthesis by enlarging the phosphocholine pool. In fact, the choline uptake of MDCK cells is stimulated 1.7-fold by PMA treatment, and HePC antagonizes this PMA-induced stimulation. In this context, it is noteworthy that HePC was also shown to inhibit

protein kinase C, another membrane-associated enzyme (Zheng et al., 1990; Geilen et al., 1991; Überall et al., 1991). Recently, it was suggested that choline uptake may be modulated by phosphorylation/dephosphorylation, but the relevant protein kinases were not identified (Hatch et al., 1991). However, an enhanced choline uptake may also arise from a decreasing phosphocholine pool via feedback regulation.

By comparing the data obtained from HeLa cells with those obtained from MDCK cells, we suggest that there is evidence for two distinct mechanisms by which PMA may stimulate PtdCho biosynthesis: (i) translocation of cytidylyltransferase to membranes and (ii) enhancement of choline uptake. HeLa cells have a large phosphocholine pool (as seen by intense labelling after 6 h of choline pulse) and a relatively small amount of membrane-bound cytidylyltransferase; after 1 min of digitonin release, only 25 % of the enzyme resides at the membrane. Therefore, the target of regulation is the cytidylyltransferase translocation process. In contrast, MDCK cells have a small phosphocholine pool (as seen by weak labelling after 6 h of choline pulse) and a relatively high amount of activated cytidylyltransferase; after 1 min of digitonin release, at least 45 % is membrane-bound. In consequence, the balanced choline uptake may be a potential target of the regulation of PtdCho biosynthesis in this cell line. Finally, it should be noted that, beside enhanced choline uptake, there might be direct activation of cytosolic cytidylyltransferase in MDCK cells, as reported for Hep G2 and alveolar type II cells (Weinhold et al., 1989).

This work was supported by a grant from the Freie Universität Berlin (Forschungsgebietsschwerpunkt 'Zelloberflächen und Erkennungsprozesse'), the Fonds der Chemischen Industrie and the Trude-Goeke-Stiftung. We thank Dr. T. A. Scott for improving the English style of the manuscript. Th. W. was recipient of a doctoral fellowship (NAFoG) of the Freie Universität Berlin.

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Received 10 August 1992/27 November 1992; accepted 4 December 1992