Selective down-regulation of protein kinase c- ϵ by carcinogens does not prevent stimulation of phospholipase D by phorbol ester and platelet-derived growth factor

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It is well established that activators of protein kinase C (PKC) also enhance the activity of phospholipase D (PLD), and that this regulatory mechanism is altered in transformed cells. Here we used the C3H/10T1/2 mouse embryo fibroblast line, a cellular model for the study of carcinogenesis, to examine possible effects of carcinogens on the PKC isoenzyme pattern and on the regulation of PLD by the PKC activators phorbol 12-myristate 13-acetate (PMA) and platelet-derived growth factor (PDGF). Treatment of these fibroblasts with 0.5 μ g/ml 7,12-dimethylbenz[*a*]anthracene or benzo[*a*]pyrene for 24 h greatly decreased (> 80%) the amount of immunoreactive PKC- ϵ . Of the remaining three isoenzymes identified, carcinogens alone had no effect on the cellular status of PKC- α and PKC- δ , although they

appeared to promote slightly PMA-induced membrane translocation of the cytosolic forms of these isoenzymes in exponentially growing cells. Carcinogens and/or PMA had no effects on the cellular content or distribution of PKC- ζ . Chronic (24 h) treatments with carcinogens resulted in increased or decreased release of [14 C]ethanolamine or [14 C]choline from the appropriate prelabelled phospholipids, respectively. However, carcinogens failed to block the stimulatory effects of PMA and PDGF on the hydrolysis of phosphatidylethanolamine and phosphatidylcholine or on the synthesis of phosphatidylethanol mediated by PLD. These data indicate that in fibroblasts PKC- ϵ is not a major regulator of PLD activity.

INTRODUCTION

Regulation of phospholipase D (PLD) activity in animal cells has recently received considerable attention because phospholipid hydrolysis by this enzyme produces phosphatidic acid, a potential second messenger in several cell types [1–6]. It is generally accepted that protein kinase C (PKC) is a major regulator of PLD (reviewed in [7,8]); however, the mechanism(s) by which such regulation occur(s) is(are) not known. Most cell types contain several PKC isoenzymes, which may have specific functions [9]. Identification of the PKC isoenzyme(s) involved in the regulation of PLD activity is an important step in understanding the regulatory mechanism itself. Recently, Eldar et al. [10] reported that overexpression of PKC- α in fibroblasts failed to alter regulation of PLD by the PKC activator phorbol 12-myristate 13-acetate (PMA). They concluded that PKC- α is not a direct regulator of PLD and suggested, instead, that PKC- ϵ may have such regulatory function. In apparent agreement with this possibility, most recently Pfeilschifter and Huwiler [11] reported that after 8 h treatment of rat renal mesangial cells with PMA, PLD still was fully active, despite complete down-regulation of PKC- α and PKC- δ , but not PKC- ϵ . On the basis of these findings, they concluded that PLD activity was regulated by PKC- ϵ [11].

In a previous study [12], we reported that 15-fold overexpression of PKC- ϵ in NIH 3T3 fibroblasts promoted the stimulatory effect of ethanol on phospholipase C-mediated hydrolysis of phosphatidylethanolamine (PtdEtn). However, subsequently we observed that PMA-induced PLD-mediated hydrolysis of PtdEtn and phosphatidylcholine (PtdCho) in PKC- ϵ overexpressors was increased only about 1.5-fold, regardless of

the length of incubation time (5–60 min) used (Z. Kiss and W. H. Anderson, unpublished work). However, the amount of PKC- ϵ in normal fibroblasts may be nearly sufficient to activate PLD fully in the presence of PMA. Clearly, to prove definitely the specific role of PKC- ϵ in the regulation of phospholipid hydrolysis, it is necessary to demonstrate that selective down-regulation of this isoenzyme prevents activation of PLD by PMA.

In fibroblasts and several other cell types, activated PLD hydrolyses both PtdCho and PtdEtn with similar efficiencies [13–19]. Interestingly, transformation of fibroblasts by *v-ras* or *Ha-ras* was found to alter PMA-stimulated hydrolysis of both PtdEtn and PtdCho [14]. This gave the idea that carcinogens may also alter phorbol ester regulation of PLD, perhaps through inducing selective changes in the PKC isoenzyme pattern.

The C3H/10T1/2 embryonic fibroblast line is a well established cellular model for the study of chemical carcinogenesis [20–22]. Thus, this cell line appeared to be ideally suited for an examination of possible effects of chemical carcinogens on the cellular content and distribution of individual PKC isoenzymes and on PLD-mediated hydrolysis of PtdCho and PtdEtn. We report here that in these fibroblasts the environmental carcinogens 7,12-dimethylbenz[*a*]anthracene (DMBA) and benzo[*a*]pyrene (B[*a*]P) selectively down-regulated PKC- ϵ , but none of these carcinogens blocked the stimulatory effects of PMA or platelet-derived growth factor (PDGF) on the hydrolysis of PtdEtn and PtdCho.

MATERIALS AND METHODS

Materials

B[*a*]P, DMBA, PMA, Dowex 50 W (H⁺ form), sphingosine,

Abbreviations used: PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PtdEtOH, phosphatidylethanol; B[*a*]P, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; PDGF, platelet-derived growth factor.

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phenylmethanesulphonyl fluoride, leupeptin and aprotinin were purchased from Sigma; biotinylated goat anti-rabbit IgG, streptavidin-alkaline phosphatase conjugate, PDGF-BB (human, recombinant), Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were bought from Boehringer Mannheim; staurosporine was from LC Services. Polyclonal antibodies raised against the α -, δ -, ζ - and ϵ -PKC isoenzymes were kindly given by Dr. Yusuf A. Hannun (Duke University, Durham, NC, U.S.A.); [1- 14 C]palmitic acid (60 mCi/mmol), [2- 14 C]ethanolamine (50 mCi/mmol) and [methyl- 14 C]choline (55 mCi/mmol) were from Amersham; tissue-culture reagents were bought from GIBCO BRL.

Cell culture

The C3H/10T1/2 embryonal fibroblast line was from the American Type Culture Collection. Fibroblast cultures were maintained in Basal Eagle Medium supplemented with 10% (v/v) fetal-calf serum (heat-inactivated). Cells were used between passages 10 and 15. At the time of harvest for analysis, cells were either 50–60% (Figures 1, 4 and 5) or 90–100% (Figures 2 and 3, and Table 1) confluent.

Western-blot analysis of PKC isoenzymes

Fibroblasts were grown up to about 50–60 or 90–100% confluency in 100 mm-diameter dishes in Basal Eagle Medium (supplemented with 10% fetal-calf serum) for 24 h in the absence or presence of 0.5 μ g/ml B[a]P or DMBA. The fibroblasts were then treated with vehicle dimethyl sulphoxide (0.01%) or PMA (100 nM) for 10 min, and scraped into homogenization buffer (on ice), containing 20 mM Tris/HCl, pH 7.5, 1 mM phenylmethanesulphonyl fluoride, 100 μ g/ml leupeptin and 25 μ g/ml aprotinin. After homogenization, homogenates were centrifuged at 15000 *g* for 20 min (at 4°C) to prepare cytosolic and particulate fractions. Separation of proteins by gel electrophoresis and subsequent Western-blot analysis of PKC isoenzymes were performed as described elsewhere [12,23]. The relative amounts of PKC isoenzymes were estimated by densitometric scanning using a Hoefer GS-300 Scanning Densitometer equipped with GS-365 Data System and Hewlett Packard Vectra CS computer.

Measurement of PLD-mediated hydrolysis of PtdCho and PtdEtn in fibroblasts

Fibroblasts were grown in 150 mm-diameter dishes in the presence of either [methyl- 14 C]choline (0.3 μ Ci/ml) or [2- 14 C]ethanolamine (0.25 μ Ci/ml) for 48 h. Fibroblasts were washed and then incubated in fresh medium for 4 h to decrease the level of water-soluble 14 C-labelled phospholipid precursors [24]. For 12 h or 24 h treatments, carcinogens were added to fibroblasts both for the last 8 h or 20 h of the labelling period respectively, and for the subsequent 4 h incubation periods in fresh medium. Labelled fibroblasts were harvested by scraping from three dishes into fresh medium containing carcinogen when appropriate. Since a 30 min period is needed after scraping for cellular 1,2-diacylglycerol concentrations to return to control levels [25], an additional 20 min incubation period between scraping and centrifugation (10 min at 500 *g*) was inserted. At this step, about 95% of fibroblasts were viable, as determined by the Trypan Blue dye exclusion assay, and they contained 700–1200 d.p.m. of [14 C]choline or [14 C]ethanolamine/ 10^6 cells. Scraped cells also retain sensitivity to phorbol ester and hormones with respect to stimulation of PLD activity [13–15]. Portions (0.2 ml) of cell suspensions [(0.9–1.0) $\times 10^6$ cells/ml] were incubated (final vol-

ume 0.25 ml) in the presence of unlabelled choline (20 mM) or ethanolamine (2 mM) (to prevent further metabolism of newly formed 14 C-labelled bases) [10,24]; the incubation medium also contained carcinogen at the appropriate concentration. Fractionation of choline and ethanolamine metabolites was performed on Dowex-50 W (H⁺)-packed columns (Bio-Rad Econo-columns; 1 ml bed volume) with minor modifications of the procedure described by Cook and Wakelam [26]. The initial flow-through (4.5 ml), along with a following 3.5 ml or 5 ml water wash, contained glycerophosphoethanolamine or glycerophosphocholine respectively. Ethanolamine phosphate and choline phosphate were eluted by 15 ml and 20 ml of water, respectively. Finally, ethanolamine and choline were eluted by 12 ml and 20 ml of 1 M HCl, respectively. The metabolites of [14 C]ethanolamine and [14 C]choline were further identified by t.l.c. [13]. Phospholipids were separated as described previously [27].

Determination of the formation of phosphatidylethanol (PtdEtOH) in fibroblasts

This was performed with [14 C]palmitic acid-labelled fibroblasts as previously described [25].

RESULTS

Effects of carcinogens and PMA on the cellular content and distribution of PKC isoenzymes

In C3H/10T1/2 fibroblasts the distribution of PKC- ϵ in the cytosolic and membrane fractions, as well as the ratio between the phosphorylated and non-phosphorylated forms of the enzyme, depends on the growth rate of fibroblasts. In exponentially growing (50–60% confluent) fibroblasts, PKC- ϵ was predominantly present in the cytosolic fraction (Figure 1, lane 1) and was represented by only one band which could be labelled with [32 P]P_i (results not shown). Treatment of these fibroblasts with 0.5 μ g/ml DMBA for 24 h decreased the total cellular content of PKC- ϵ by about 80% as determined by densitometric scanning (Figure 1, lanes 3, 4). Addition of PMA to control fibroblasts for 10 min induced both partial down-regulation of PKC- ϵ and translocation of the remaining enzyme molecules

Figure 1 Western-blot analysis of PKC- ϵ in control and DMBA-treated fibroblasts

Treatment of C3H/10T1/2 fibroblasts with 0.5 μ g/ml DMBA for 24 h, subsequent treatments with 100 nM PMA for 10 min, preparation of subcellular fractions, and analysis of PKC- ϵ was performed as indicated in the Materials and methods section. At the time of harvest, fibroblasts were 50–60% confluent, determined by visual inspection and by counting the cell number. Lanes I and II represent molecular-mass standards (kDa) and a partially purified rat brain standard, respectively. The arrow indicates the position of PKC- ϵ . Fibroblasts were initially untreated (lanes 1, 2 and 5, 6), or were treated with DMBA (lanes 3, 4 and 7, 8), followed by incubation of fibroblasts for 10 min in the absence (lanes 1–4) or presence of 100 nM PMA (lanes 5–8). Lanes 1, 3, 5, 7 were loaded with 75 μ g of soluble-fraction protein, and lanes 2, 4, 6, 8 were loaded with 75 μ g of particulate-fraction protein. This experiment was repeated three times with similar results.

Figure 2 Time- and concentration-dependence of DMBA-induced down-regulation of PKC- ϵ

C3H/10T1/2 fibroblasts were treated for 12 h (a, b) or 24 h (c, d) with the following concentrations ($\mu\text{g/ml}$) of DMBA; 0 (lanes 1, 2), 0.05 (lanes 3, 4), 0.1 (lanes 5, 6) and 0.5 (lanes 7, 8). After DMBA treatment, fibroblasts were incubated for a further 10 min in the absence (a, c) or presence of 100 nM PMA (b, d). At the time of harvest, fibroblast cultures were 90–100% confluent. Lanes 1, 3, 5, 7 were loaded with 75 μg of soluble-fraction protein, and lanes 2, 4, 6, 8 were loaded with 75 μg of particulate-fraction protein. Lane 1 represents the rat brain standard. The arrow indicates the position of PKC- ϵ . This experiment was repeated twice with similar results.

Figure 3 Western-blot analysis of PKC- ϵ in B[a]P-treated fibroblasts

Fibroblasts were untreated (lanes 1, 2) or were treated with 0.5 $\mu\text{g/ml}$ B[a]P for 24 h (lanes 3, 4). At the time of harvest, fibroblast cultures were 90–100% confluent. Lane 1 represents rat brain standard. Lanes 1, 3 and 2, 4 are cytosolic and particulate fractions respectively. The arrow indicates the position of PKC- ϵ . This experiment was repeated three times with similar results.

from the cytoplasmic (Figure 1, lane 5) to the membrane fraction (Figure 1, lane 6). DMBA did not appear to interfere with PMA-induced membrane translocation of the small amount of PKC- ϵ which remained in the fibroblasts after carcinogen treatment (Figure 1, lanes 7, 8). We still should note here that, with each PKC-isoenzyme-specific antibody examined here (see also Figure 4), we always observed an abundant 120–125 kDa band. The

identity of this clearly non-specific band is at present unknown; we only determined that it derives from the preparation of streptavidin–alkaline phosphate conjugate.

In fibroblast cultures present in the slow growth phase (90–100% confluent), PKC- ϵ was still predominantly present in the cytosolic fraction (Figure 2a, lane 1), but a significant portion of enzyme molecules was also present in the membrane fraction (Figure 2a, lane 2). In contrast with rapidly growing fibroblasts, in these fibroblasts PKC- ϵ was often (but not always; see Figure 3) represented by two, slower and faster moving, bands. Only the upper band could be labelled with [^{32}P]P $_i$ (results not shown). When fibroblasts were treated with 0.5 $\mu\text{g/ml}$ DMBA for only 4 h, there was less than 25% decrease in the cellular content of PKC- ϵ (results not shown). However, treatment of fibroblasts for 12 h with 0.05 (Figure 2a, lanes 3, 4), 0.1 (Figure 2a, lanes 5, 6) or 0.5 $\mu\text{g/ml}$ DMBA (Figure 2a, lanes 7, 8) resulted, in each case, in greater than 60% decreases in the cellular content of PKC- ϵ . It is important to note that at this time point PKC- ϵ was still detectable, even in the presence of the highest concentration of DMBA. Treatment of fibroblasts with 100 nM PMA for 10 min specifically enhanced the membrane content of the slower moving (phosphorylated) form of PKC- ϵ (Figure 2b, lanes 1, 2). Treatment of cells with DMBA for 12 h before the addition of PMA did not interfere with PMA-induced translocation of remaining PKC- ϵ (Figure 2b, lanes 3–8).

When fibroblasts were treated with DMBA for 24 h and then harvested in the near-confluent state, we observed complete elimination of PKC- ϵ by the highest (0.5 $\mu\text{g/ml}$) concentration of carcinogen (Figure 2c, lanes 7, 8). Again, lower concentrations of carcinogens failed to modify the effect of PMA on membrane translocation of the remaining PKC- ϵ molecules (Figure 2d, lanes 3–6).

When near-confluent fibroblasts were treated with 0.5 $\mu\text{g/ml}$ B[a]P for 24 h, cellular PKC- ϵ was decreased below the detection limit (Figure 3). At a lower dose (0.1 $\mu\text{g/ml}$) of B[a]P, which is sub-optimal with respect to inducing cell transformation [20], this carcinogen induced about 50% decrease in the cellular content of PKC- ϵ after 24 h treatment. Although a detailed time course of B[a]P action has not yet been analysed, a treatment with 0.5 $\mu\text{g/ml}$ B[a]P for 4 h yielded less than 25% decrease in the cellular content of PKC- ϵ (results not shown).

Of the nine other PKC isoenzymes which have been described in various cell types so far [9], in these fibroblasts we have detected the α , δ and ζ isoenzymes. In control (~ 50 –60% confluent) fibroblasts, about 90% of PKC- α was present in the cytosolic fraction (Figure 4a, lanes 1, 2). Chronic (24 h) treatment of fibroblasts with 0.5 $\mu\text{g/ml}$ DMBA failed to cause any change in the cellular content or distribution of this enzyme (Figure 4a, lanes 3, 4). A short (10 min) treatment of control fibroblasts with PMA (100 nM) resulted in both partial down-regulation (about 30–40% of total cellular pool) and membrane translocation of the remaining PKC- α molecules (Figure 4a, lanes 5, 6). Pretreatment of fibroblasts with DMBA consistently caused an apparent increase in PMA-induced translocation of PKC- α (~ 1.8 -fold; $n = 4$) (Figure 4a, lanes 7, 8). Presumably, carcinogen treatment decreased the ability of membranes to down-regulate translocated PKC- α .

In control fibroblasts, the major portion of PKC- δ was also present in the cytosolic fraction, and this pattern of distribution was not altered by DMBA treatment (Figure 4b, lanes 1 and 2 compared with lanes 3 and 4). In control fibroblasts, PMA rapidly induced membrane translocation of PKC- δ (Figure 4b, lanes 5, 6). Again, pretreatment of fibroblasts with DMBA enhanced the ability of PMA to increase the membrane content of PKC- δ about 1.6-fold ($n = 4$) (Figure 4b, lanes 7, 8). Treat-

Figure 4 Western-blot analysis of α -, δ - and ζ -PKC isoenzymes in control and DMBA-treated fibroblasts

Treatments of fibroblasts with 0.5 $\mu\text{g/ml}$ DMBA for 24 h, subsequent treatments with 100 nM PMA for 10 min, and analysis of PKC- α (a), PKC- δ (b) and PKC- ζ (c) were performed as described in the Materials and methods section. At the time of harvest, fibroblast cultures were 50–60% confluent. Lane 1 represents rat brain standard. The arrows indicate the position of the respective PKC isoenzymes. Fibroblasts were initially untreated (lanes 1, 2 and 5, 6) or were treated with DMBA (lanes 3, 4 and 7, 8), followed by incubation of fibroblasts for 10 min in the absence (lanes 1–4) or presence of 100 nM PMA (lanes 5–8). Lanes 1, 3, 5, 7 and 2, 4, 6, 8 are cytosolic and particulate fractions respectively. This experiment was repeated three times with similar results.

ments with 0.5 $\mu\text{g/ml}$ B[a]P mimicked the potentiating effects of DMBA on PMA-induced membrane translocation of both PKC isoenzymes (results not shown).

PKC- ζ , in contrast with other PKC isoenzymes, does not bind PMA and does not respond to PMA in any other conventional way [28–31]. Chronic (24 h) treatments of fibroblasts with DMBA (or B[a]P) and/or shorter (10 min) treatments with PMA also failed to elicit any noticeable change in the cellular content or distribution of PKC- ζ (Figure 4c).

In view of the observation that carcinogens more completely down-regulated PKC- ϵ in the slowly growing fibroblasts, it was decided to examine the effects of DMBA on the α -, δ - and γ -PKC isoenzymes in near-confluent fibroblast cultures. Treatment of these (90–100% confluent) cultures with 0.5 $\mu\text{g/ml}$ DMBA for 24 h also failed to decrease significantly the cellular content of these PKC isoenzymes (results not shown).

Effects of carcinogens and PMA on PLD-mediated hydrolysis of PtdEtn and PtdCho

The above observations, that in the slow- (or no-) growth phase carcinogens can selectively down-regulate PKC- ϵ , made carcinogen-treated fibroblasts an ideal choice to explore the role of PKC- ϵ in the regulation of PLD activity. Because the hydrolysis of PtdEtn and PtdCho is oppositely affected by cell transformation [14], it appeared more informative to examine first the effects of carcinogens on the hydrolysis of individual phospho-

Figure 5 Effects of chronic carcinogen treatments on PMA-stimulated hydrolysis of PtdEtn and PtdCho in fibroblasts

Attached C3H/10T1/2 fibroblasts were labelled with [^{14}C]ethanolamine (a) or [^{14}C]choline (b) for 48 h and were either untreated (●) or treated with 0.5 $\mu\text{g/ml}$ DMBA (▲) or B[a]P (■) during the last 24 h of the labelling period. Preparations of suspended fibroblasts, derived from labelled confluent cultures, were then treated with the indicated concentrations of PMA for 30 min. The average ^{14}C content of the PtdEtn and PtdCho pools in these experiments was $(1.06\text{--}1.24) \times 10^6$ and $(7.65\text{--}8.84) \times 10^5$ dpm/ 10^6 cells respectively. Data are means $\pm$ S.E.M. of four independent incubations. Similar results were obtained in two other experiments each performed in triplicate.

lipids, instead of analysing the PLD-mediated transphosphatidyl-ation reaction, which does not discriminate among phospholipid substrates. First, we performed experiments with [^{14}C]ethanolamine- and [^{14}C]choline-labelled attached fibroblasts. We detected significant stimulatory effects of PMA on phospholipid hydrolysis in both untreated and carcinogen-treated fibroblasts (results not shown). However, the background levels of [^{14}C]choline (3000–4000 d.p.m./ 10^6 cells) and [^{14}C]ethanolamine (7000–8000 d.p.m./ 10^6 cells) were too high to allow proper evaluation of [^{14}C]choline and [^{14}C]ethanolamine formation in the absence of PMA. Therefore, in the following experiments we used suspended fibroblasts (derived from confluent cultures), which contained much lower background levels of [^{14}C]choline and [^{14}C]ethanolamine (see the Materials and methods section), and they also responded to PMA with respect to stimulation of PLD. As shown in Figure 5(a), treatment of fibroblasts with 0.5 $\mu\text{g/ml}$ B[a]P or DMBA slightly (1.5–1.7-fold) enhanced formation of [^{14}C]ethanolamine from the prelabelled cellular PtdEtn pool. Importantly, treatments with either DMBA or B[a]P did not prevent the stimulatory effect of PMA on PtdEtn hydrolysis, although carcinogen treatment decreased the fold stimulatory effects of 5–100 nM PMA by 20–34%. In contrast with PtdEtn hydrolysis, PtdCho hydrolysis was inhibited by about 25–30% in the carcinogen-treated fibroblasts (Figure 5b). However, carcinogens failed to change appreciably the fold stimulatory effects of PMA on the formation of [^{14}C]choline (Figure 5b). We should note here that, at the conclusion of PLD assay, PKC- ϵ was completely absent from carcinogen-treated fibroblasts, as determined in subsequent experiments (results not shown).

Formation of PtdEtOH appears to be a specific function of PLD. To confirm that PMA-induced phospholipid hydrolysis

Table 1 Comparison of the effects of PMA, PDGF, sphingosine and staurosporine on the formation of PtdEtOH in [¹⁴C]palmitate-labelled fibroblasts

C3H/10T1/2 fibroblasts were labelled with [¹⁴C]palmitic acid for 24 h in the absence (Untreated) or presence of 0.5 µg/ml DMBA (DMBA-treated). Confluent fibroblast cultures were washed, and then suspended labelled fibroblasts were incubated for 30 min in the absence or presence of PMA (100 nM), PDGF-BB (50 ng/ml), sphingosine (50 µM) or staurosporine (5 µM) as indicated. In each case, the incubation medium contained 200 mM ethanol. The ¹⁴C content of PtdCho and PtdEtn was 735 000 and 189 000 d.p.m./10⁶ cells. Data are means ± S.E.M. of four incubations. This experiment was repeated once with similar results.

Addition	Formation of [¹⁴ C]PtdEtOH (d.p.m./30 min per 10 ⁶ cells)	
	Untreated	DMBA-treated
None	1490 ± 310	2060 ± 260
PMA	16840 ± 1490	19820 ± 1820
PDGF	5520 ± 610	6390 ± 700
Sphingosine	10290 ± 990	10330 ± 830
Staurosporine	4510 ± 380	4950 ± 430

indeed involved PLD, next we examined the effects of PMA on PtdEtOH formation in [¹⁴C]palmitic acid-labelled fibroblasts incubated in the presence of 200 mM ethanol. In the absence of PMA, both control and DMBA-treated (0.5 µg/ml; 24 h) fibroblasts produced only relatively small amounts of [¹⁴C]PtdEtOH (Table 1). In control and DMBA-pretreated fibroblasts, 100 nM PMA (30 min) stimulated PtdEtOH formation 11.3- and 9.6-fold respectively. PDGF, which in NIH 3T3 fibroblasts stimulates the hydrolysis of both PtdEtn and PtdCho by a PKC-dependent mechanism [15], also stimulated the formation of PtdEtOH in untreated and carcinogen-treated fibroblasts 3.7- and 3.1-fold, respectively (Table 1). Of the other stimulators of PLD examined, carcinogen treatment also slightly decreased the stimulatory effects of sphingosine and staurosporine (Table 1). These data confirm that carcinogens do not prevent, although they may slightly decrease activation of PLD by non-hormonal and hormonal activators of PKC and by other, PKC-independent, activators.

DISCUSSION

A major finding of this work is that chronic (24 h) treatment of fibroblasts with 0.5 µg/ml DMBA or B[a]P resulted in practically complete and selective down-regulation of PKC-ε if fibroblasts were in the near-confluent or confluent state at the time of harvest. These carcinogens also dramatically, but not completely, decreased the cellular content of PKC-ε in rapidly growing (50–60% confluent) fibroblast cultures. The reason for the slightly different effects of carcinogens in confluent and non-confluent cells is at present unknown.

Since carcinogens down-regulated only PKC-ε, and this isoenzyme was considered as a potential regulator of PLD [10,11], it was of interest to determine the effects of PKC activators on PLD-mediated hydrolysis of phospholipids in carcinogen-treated cells. Somewhat unexpectedly, complete down-regulation of PKC-ε by carcinogens had only a relatively small impact on PMA-stimulated PLD activity. Carcinogens appeared to decrease PtdCho hydrolysis slightly in the absence of PMA, but the fold stimulatory effects of PMA were unaffected. In contrast, the carcinogens had small stimulatory effects on PtdEtn hydrolysis in the absence of PMA, and they also decreased the fold

stimulatory effects of different concentrations of PMA by 20–34%. Chronic treatments with DMBA also slightly (~15%) decreased PLD-mediated formation of PtdEtOH in the presence of PMA or PDGF. This most probably reflects a decrease of PMA effect on PtdEtn hydrolysis, because carcinogens did not alter the fold stimulatory effects of PMA on PtdCho hydrolysis. Whether or not these small stimulatory and inhibitory effects of carcinogens are related to the loss of PKC-ε remains to be established. Since carcinogens also slightly decreased the effects of sphingosine and staurosporine, agents which are unlikely to act through the stimulation of the regulatory PKC isoenzyme, even the carcinogen-induced small decreases in the effects of PMA and PDGF may be unrelated to down-regulation of PKC-ε.

Our finding that PKC-ε does not play a major role in the regulation of PLD activity contrasts with the conclusion reached by Pfeilschifter and Huwiler [11]. These authors reported [11] that treatment of rat mesangial cells with PMA for 8 h resulted in full activation of PLD activity, despite complete down-regulation of PKC-α and PKC-δ. Since under the same condition PKC-ε was not down-regulated, Pfeilschifter and Huwiler [11] concluded that this isoenzyme regulates PLD activity. This suggests that in different cell types different PKC isoenzymes may regulate PLD activity. Alternatively, in both cell lines regulation of PLD by PMA may involve an at present unidentified phorbol-ester-binding protein. Although further experiments are required to distinguish between these possibilities, we also observed that 15-fold overexpression of PKC-ε in NIH 3T3 fibroblasts [12] enhanced the stimulatory effects of 5–40 nM PMA on PLD activity about 1.5-fold (Z. Kiss and W. H. Anderson, unpublished work). Although this does not necessarily mean that PKC-ε is a direct regulator of PLD, at present we cannot exclude the possibility that a minor pathway of PLD regulation indeed involves PKC-ε.

These experiments are not suitable to determine the possible regulatory role of the other PKC isoenzymes present in fibroblasts. PKC-α is a major isoenzyme in these cells, and therefore it may not be the rate-limiting factor in activating PLD. Therefore, the small potentiating effects of carcinogens on PMA-induced membrane translocation of PKC-α may not necessarily lead to increased activity of PLD, even if this isoenzyme is the major regulator of phospholipid hydrolysis. The same consideration may be applicable concerning the possible regulatory role of PKC-δ. On the basis that PKC-ζ does not respond to PMA in any conventional way [28–31], at present we do not consider this isoenzyme as a possible regulator of PLD.

The mechanism by which carcinogens decrease the cellular content of PKC-ε is at present unknown. In principle, inhibition of synthesis of PKC-ε or stimulation of its degradation are the most likely mechanisms that may be involved in this action of carcinogen. Examination of both possible mechanisms is at present underway in our laboratory.

In other studies, PKC-ε was shown to assume oncogenic properties when overexpressed in fibroblasts [32,33]. This would suggest that up-regulation of this enzyme may be required for neoplastic transformation. However, the present finding that carcinogens down-regulate PKC-ε appears to be incompatible with this hypothesis. In fact, it is quite possible that chemical carcinogenesis involves down-regulation of PKC-ε. Clearly, more work is required to clarify the role, if any, of PKC-ε in carcinogenesis.

In summary, carcinogens were shown to down-regulate PKC-ε specifically. The role of this phenomenon in chemical carcinogenesis remains to be established. Down-regulation of PKC-ε led only to a relatively small decrease (PtdEtn hydrolysis) or no

decrease at all (PtdCho hydrolysis) in PMA-stimulated PLD activity. These data establish that in fibroblasts PKC- ϵ is not a major mediator of the effect of PMA on PLD activity.

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