Differential effects of phorbol ester on growth and protein kinase C isoenzyme regulation in human hepatoma Hep3B cells

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PMA has both mitogenic and antiproliferative effects on human hepatoma Hep3B cells. In response to low PMA concentration (10 nM), Hep3B cells displayed an increasing proliferation potentiation. At high PMA concentration (1 μ M) Hep3B cells exhibited modest cytostatic effects. Determinations of protein kinase C (PKC) activity in PMA-treated cells revealed that alterations in PKC activity are associated with proliferative capacity. The decrease in PKC activity mediated by a high dose of PMA was accompanied by cell growth inhibition. Increases in PKC activity mediated by a low dose of PMA were consistent with proliferation stimulation. Immunoblot analysis showed that there are at least six PKC isoenzymes: α , δ , ϵ , μ , ζ and ι/λ ,

constitutively expressed in Hep3B cells. Cellular fractionation and immunocytochemical staining results demonstrated that both 10 nM and 1 μ M PMA treatments induced a marked translocation of PKC- α from cytosol to membrane or nuclear fraction within 5–30 min. At the same time PKC- δ and ϵ were translocated from the membrane to nuclear fraction. In addition, prolonged treatment with 1 μ M PMA, but not with 10 nM PMA, selectively mediated the down-regulation of these three PKC isoenzymes. The distinct effects of different concentrations of PMA on cell proliferation and PKC- α , δ and ϵ isoenzyme modulation support the involvement of these three PKC isotypes in the mechanism of action of Hep3B cells in cell growth events.

INTRODUCTION

Protein kinase C (PKC), in the family of serine/threonine protein kinases, is involved in one of the major signal transduction systems and is activated on the external stimulation of cells by various ligands, including hormones, mitogens and neurotransmitters [1]. So far at least 11 isoenzymes of PKC have been identified; they have been divided into three major groups on the basis of their biochemical and structural properties. The conventional PKCs (cPKC- α , β I, β II and γ) are regulated by phosphatidylserine, diacylglycerol and Ca^{2+} . The novel PKCs (nPKC- δ , ϵ , η , θ and μ) are Ca^{2+} -independent because they lack the Ca^{2+} -binding C2 domain. The atypical PKCs (aPKC- ζ and ι ; aPKC- ι is the human counterpart of mouse PKC- λ) are not activated by phorbol ester or diacylglycerol [1–3].

PMA and other phorbol esters are potent activators of PKC. PMA mimics the effects of PKC's natural activator, diacylglycerol [1,4,5]. It is widely recognized that phorbol esters are promoters of skin tumours, with pleiotropic and often tissue-specific cellular responses such as proliferation [6], differentiation [7], apoptosis [8] and gene expression [9]. Cellular studies with phorbol esters have revealed that phorbol ester-mediated cellular responses occur via a PKC-dependent signal transduction pathway [1–4]. Emerging data on the structure, biochemical properties and tissue distribution of the individual PKC family members have suggested that different isoforms might execute distinct cellular functions, and more than one isoform is usually expressed within a particular cell type [1–3]. It is therefore plausible that the nature of the cellular response is determined by the differential expression of specialized isoenzymes in individual cell types, probably at different cellular locations. The determination of

specific biological roles for each PKC isoform, as well as the discovery of whether these roles are the same in all cell types, has been complicated by the large size of this family and by the fact that most cell types express only a few of the isoenzymes. Few biological functions have therefore been defined for particular isoforms. PKC- α and δ , but not β II ϵ , η or ζ , mediate the differentiation of mouse myeloid 32D cells into mature macrophages [10]. Overexpression of the aPKC- ζ isoform results in the deregulation of growth control in mouse fibroblasts [11]. nPKC ϵ overexpression in NIH/3T3 cells leads to oncogenic transformation [12], whereas PKC- δ inhibits proliferation [13]. These findings have led us and others to hypothesize that the wide spectrum of functions ascribed to PKCs *in vivo* is achieved by a family of functionally similar enzymes, with isoform-specific differences in subcellular localization.

Many studies have suggested that signals mediated via PKC isoforms have a role in the process of tumour promotion [14–18] and also in the action of various growth factors [19–21] and oncogenes [22]. There is now extensive evidence to indicate that tumours develop secondarily to abnormalities in PKC-mediated signal transduction [17,18]. Thus PKC expression is a potential marker for malignant diseases [23–26]. Because PKC is a large family of proteins and most cells contain more than one isoform, the pattern and the expression of individual isoforms vary between tissues and cell lines [3,5]. Shifts in isoenzyme pattern and expression might well be part of the signal transduction alterations that affect the biological response of neoplastic cells. Recently the PKC-dependent mechanism of tumour development in colon cancer has been characterized: the various PKC isoforms seemed to be differentially activated and down-regulated and to have different effects on growth regulation [27,28]. However,

Abbreviations used: MARCKS, myristoylated alanine-rich C-kinase substrate; PKC, protein kinase C; aPKC, atypical protein kinase C, cPKC, conventional protein kinase C; nPKC, novel protein kinase C.

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very little is known about the differential functions of individual PKC isoforms in pathways of signal transduction and growth regulation in human hepatocarcinoma cells. To define the possible role of PKC in the modulation of cell proliferation in human hepatocarcinoma cells, we assessed the changes in PKC activity, isotype pattern and subcellular distribution of PMA-treated human hepatoma Hep3B cells. Our results demonstrate that there are at least six PKC isoforms, PKC- α , δ , ϵ , μ , ζ and ι/λ , constitutively expressed in this cell line. The down-regulation of PKC- α , δ and ϵ in response to PMA is associated with a decrease in PKC activity and an inhibition of cell growth. This finding shows that PMA-induced growth is accompanied by the differential regulation of PKC isoenzymes in Hep3B cells.

MATERIALS AND METHODS

Drugs and reagents

PMA was purchased from Sigma. Stock solutions of 1 mM PMA were prepared in ethanol and stored at -20°C . The final concentration of ethanol did not exceed 0.1 %, which on its own did not affect cell growth. The monoclonal antibodies against specific PKC isoenzymes α , β , δ , ϵ , μ , θ , ζ , ι and λ were purchased from Transduction Laboratory; anti-(PKC- γ) antibody was from Gibco BRL, and anti-(PKC- η) antibody was from Santa Cruz. [*methyl*- ^3H]Thymidine (specific radioactivity 6.7 Ci/mmol) and [γ - ^{32}P]ATP (specific radioactivity 3000 Ci/mmol) were obtained from NEN-DuPont.

Cell culture and proliferation assay

Human hepatoma Hep3B cells were maintained in Dulbecco's modified Eagle's medium (Gibco), supplemented with 10 % (v/v) fetal calf serum and antibiotics, in a humidified air/ CO_2 (19:1) atmosphere at 37°C . The medium was changed every 2 days. The proliferation assay was performed as follows. Cells were incubated at a density of 10^5 cells per well in 12-well plates, in the presence of various concentrations of PMA in serum-free medium. The control group was treated with vehicle (0.1 % ethanol). The medium was changed and cell numbers were counted at the indicated time points, by Trypan Blue dye exclusion, with a haemocytometer.

Thymidine incorporation

The cells were treated with or without PMA (10 nM or 1 μM) at indicated time points (24, 48 and 72 h) followed by the addition of 1 μCi of [*methyl*- ^3H]thymidine per well for 4 h. After incubation, cells were rinsed with PBS, then fixed and washed with 10 % (w/v) trichloroacetic acid. The DNA was solubilized at room temperature for 20 min with 200 μl of 0.1 M NaOH. [^3H]Thymidine incorporation into DNA was measured by a liquid-scintillation counter.

Protein analysis by Western blotting

To analyse the protein expression of the PKC isoforms, the cultured cells were washed twice with PBS, then lysed with protein lysis buffer [50 mM Tris/HCl (pH 7.4)/2 mM EDTA/2 mM EGTA/150 mM NaCl/1 mM PMSF/10 $\mu\text{g}/\text{ml}$ leupeptin/10 $\mu\text{g}/\text{ml}$ aprotinin/10 $\mu\text{g}/\text{ml}$ trypsin inhibitor/1 mM NaF/1 mM sodium orthovanadate/1 % (v/v) 2-mercaptoethanol/1 % (v/v) Nonidet P40/0.3 % sodium deoxycholate]. The cell lysates were centrifuged at 100 000 g for 30 min at 4°C . Then the supernatant was collected and the concentration of protein determined by the Bradford method [29]. Next, sample lysates

were separated by SDS/PAGE [8 % (w/v) gel] and transferred electrophoretically to PVDF membrane (Millipore). The membrane was blocked with 5 % (w/v) non-fat dried milk in TBST buffer [20 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.1 % (v/v) Tween 20] and incubated overnight at 4°C in the specific anti-PKC antibody (Transduction Laboratory). The membrane was next washed with TBST and incubated with the appropriate secondary antibody. Finally the membrane was detected by exposure to film with an enhanced chemiluminescence assay kit (Amersham).

Cell treatment and subcellular fractionation

Cells were cultured in serum-free medium containing 10 nM PMA, 1 μM PMA or vehicle (control), for various periods. Cytosolic, membrane (meaning the membranes and cell organelles) and nuclear fractions of cells were obtained by previously described procedures, with some modifications [30]. In brief, after removal of the medium, cells were washed with cold PBS, scraped from the plates and allowed to swell in 0.5 ml of buffer I [50 mM Tris/HCl (pH 7.4)/2 mM EDTA/2 mM EGTA/1 mM dithiothreitol/10 $\mu\text{g}/\text{ml}$ leupeptin/10 $\mu\text{g}/\text{ml}$ aprotinin/10 $\mu\text{g}/\text{ml}$ trypsin inhibitor/1 mM PMSF/1 mM NaF/1 mM sodium orthovanadate/0.1 % (v/v) Triton X-100]. The cells were homogenized with 20 strokes of a Dounce homogenizer with pestle A. Cell fractions (cytosol, membrane and nucleus) were obtained by two-step centrifugation. The homogenates were first centrifuged (at 600 g and 4°C for 20 min) to obtain a crude nuclear pellet and a soluble fraction. The soluble fraction was further centrifuged (at 100 000 g for 30 min) to yield a cytosolic supernatant and a plasma membrane pellet, which was solubilized in buffer I containing 1 % (v/v) Triton X-100. The crude nuclear pellet was washed with buffer I, layered over 45 % (w/v) sucrose, centrifuged (at 1660 g and 4°C for 30 min), resuspended and sonicated in buffer I containing 1 % (v/v) Triton X-100. The purity of the nuclear preparations was determined by electron microscopy and monitored routinely by phase-contrast microscopy after staining with Methylene Blue. For the PKC activity assay, the total cell homogenate, purified nuclear fraction and plasma membrane-rich fractions were solubilized with 1 % (w/v) CHAPS on ice for 2 h. Protein concentration was determined by the Bradford method [29].

PKC activity assay

PKC activity was measured with a commercially available kit (Promega). In brief, the reaction was initiated by the addition of protein samples to a reaction mixture (total volume 50 μl) containing 20 mM Tris/HCl, pH 7.5, 0.4 mM CaCl_2 , 10 mM MgCl_2 , 0.5 μCi of [γ - ^{32}P]ATP (3000 Ci/mmol), 25 μM ATP, 8 μM PKC biotinylated peptide substrate, 16 μg of phosphatidylserine, 1.6 μg of diacylglycerol, 0.1 mM EDTA and 0.1 mM EGTA in the reaction mixture contributed by the protein sample. After incubation at 30°C for 10 min, reactions were terminated by adding 25 μl of 7.5 M guanidinium chloride. Samples were collected on a streptavidin disk, then washed with 1 M NaCl and water; radioactivity was measured by liquid-scintillation counting. The enzyme activity of PKC was determined by subtracting the activity of the enzyme in the absence of phospholipids (control buffer) from that of the enzyme in the presence of phospholipids (activation buffer). The value obtained with this kit is the sum of the activities of phospholipid-dependent, Ca^{2+} -dependent and Ca^{2+} -independent PKC isoenzymes activated by PMA, which transferred a γ -phosphate group of [^{32}P]ATP to a PKC-specific substrate. Kinase activity was calculated as pmol of [γ - ^{32}P]ATP incorporated/min per mg of protein: [(radioactivity

incorporated in reaction with phospholipids) – (radioactivity incorporated in reaction without phospholipids)] divided by [(time in minutes) $\times$ (mg of protein in reaction) $\times$ (specific radioactivity of [γ - 32 P]ATP)].

Immunofluorescence staining

Cells were plated on sterile slides and treated with PMA at various time points. After treatment, slides were washed with PBS and fixed with 2% (v/v) formaldehyde in PBS for 30 min. The fixed cells were washed twice with PBS, permeabilized for 3 min with acetone at -20°C , then washed twice with PBS. For indirect immunofluorescence, cover slides were incubated for 60 min at 37°C with mouse anti-(human PKC) antibodies. The slides were then washed with PBS for 15 min at room temperature and incubated for another 60 min with FITC-conjugated goat anti-mouse IgG) antibody. Finally the slides were washed extensively in PBS and mounted on objective microscope slides.

RESULTS

Effect of PMA on cell growth and morphology

It has previously been shown that PMA elicits highly cell-type-specific effects on cell proliferation, even when administered to closely related cell lines. We examined the effects of varying PMA concentrations on the cell proliferation of human hepatoma Hep3B cells. After exposure to a low dose (10 nM) of PMA for 72 h, Hep3B cells displayed an enhanced proliferative rate as assessed by an increase in cell number (Figure 1, upper panel). However, cells treated with a high dose (1 μM) of PMA for 72 h exhibited a moderate cytostatic property: the treated cells proliferated markedly more slowly than untreated control cells (Figure 1, upper panel). Consistent with the proliferation assay by Trypan Blue dye exclusion, [^3H]thymidine incorporation results showed $168.8 \pm 10.6\%$, $157.9 \pm 9.4\%$ and $127.6 \pm 12.3\%$ increases in DNA synthesis after exposure to 10 nM PMA for 24, 48 and 72 h respectively. However, 72 h after treatment, 1 μM PMA exerted a significant inhibition of DNA synthesis, an approx. 50% decrease, in comparison with the control (Figure 1, lower panel). These results demonstrate that PMA at different dosages exerts biphasic regulation on Hep3B cell proliferation. As shown in Figure 2, PMA-treated cells demonstrate transient morphological changes in comparison with the vehicle control. Cells exposed to PMA for 0.5 h adhered to plastic in a spherical configuration (Figure 2) that was quite different from the dendritic morphology displayed by the control; this PMA-mediated morphological alteration was observed continuously over an 8 h incubation period. However, the cells reverted to a normal appearance (dendritic phenotype) 24 h after the addition of PMA (Figure 2). These results suggest that the PMA-mediated phenotypic alteration is not correlated with growth potential, because 10 nM and 1 μM PMA-treated cells exhibited similarities in phenotypic alteration but differences in growth rates.

Expression of PKC isoforms in Hep3B cells

It is considered that the PMA-mediated cellular response is intrinsically linked to PMA's propensity to activate PKC. We therefore studied how the PKC isoenzymes are expressed in Hep3B cells. Total cell extracts were prepared and characterized with PKC isoform-specific antibodies. Western blot analysis showed that there are at least seven PKC isoforms expressed in Hep3B cells. Antibodies directed against PKC- α and μ specifically recognized single proteins of approx. 80 and 115 kDa respectively (Figure 3). Consistent with a previous report on R6 rat embryo

Figure 1 Effect of PMA on the proliferation of serum-deprived Hep3B cells

Upper panel: cells were cultured in 12-well plates at a density of 10^5 cells per well in serum-free Dulbecco's modified Eagle's medium. After 24 h the cells were cultured in 0.1% (v/v) vehicle medium ($\square$), 10 nM PMA ($\circ$) or 1 μM PMA ($\triangle$). The viable cell number was determined at the indicated time points (8, 24, 48 and 72 h). Lower panel: thymidine incorporation. Cells were cultured for 24, 48 or 72 h in serum-free medium lacking PMA or containing PMA at 10 nM or 1 μM . After incubation, cells were treated with [^3H]thymidine for 4 h. The amount of [^3H]thymidine incorporation was subsequently determined. Results were calibrated by radioactivity (c.p.m.)/mg of protein and normalized to the control (100%). Each point is the mean $\pm$ S.D. for nine replicates from three independent experiments.

fibroblasts [31], the anti-(PKC- δ) and anti-(PKC- ϵ) antibodies detected double bands at 74 and 76 kDa and at 85 and 89 kDa respectively (Figure 3). The anti-(PKC- λ) and anti-(PKC- ι) antibodies recognized two proteins of approx. 70 and 120 kDa and 70 and 120 kDa respectively. The anti-(PKC)- ζ antibody recognized proteins at 50, 72 and 75 kDa. However, PKC- β , γ , θ and η could not be detected in Hep3B cells.

Differential regulation of PKC isoforms by treatment with PMA

To define more precisely the role that PKC has in modulating growth rate after exposure to PMA, we measured the total content of cellular PKC isoforms in PMA-treated Hep3B cells at different time points by a time-course immunoblotting analysis. As indicated in Figure 4, treatment with 1 μM PMA for up to 8 h resulted in the complete down-regulation of the total amounts of PKC- α and δ . Long-term treatments (24, 48 and 72 h) resulted in a consistent down-regulation of these PKC isoenzymes. It should

Figure 2 Transient morphological changes in PMA-treated Hep3B cells
Cells were untreated, or treated with 10 nM or 1 μM PMA for 0.5, 8 or 24 h, then examined by phase-contrast microscopy. Magnification × 160.

Figure 3 Identification of PKC isoforms expressed in Hep3B cells
Whole cell lysate was separated by SDS/PAGE [8% (w/v) gel]; PKC isoenzymes were identified by Western blotting analysis with specific anti-(PKC isoform) antibodies as described in the Materials and methods section. The positions of molecular mass markers are indicated (in kDa) at the left.

be noted that PKC-ε expression in Hep3B cells was subject to down-regulation after 8 h of exposure to 1 μM PMA; complete down-regulation was observed at 48 to 72 h PMA incubation (Figure 4). Treatment with 10 nM PMA for various time periods exerted no significant effect on total PKC-α, δ or ε when compared with vehicle control. The results revealed the differential regulation of PKC-α, δ and ε in Hep3B cells by different concentrations of PMA. The experiments also indicated that PKC-μ, ζ, λ and ι in human hepatoma cells are resistant to down-regulation by treatment with PMA (results not shown), because the total

Figure 4 Effects of PMA on the expression of PKC isoforms at various time points in Hep3B cells

Cultured Hep3B cells were incubated with PMA (1 μM or 10 nM) for the indicated durations (0.5, 2, 4, 8, 24, 48 and 72 h). At the end of the incubation period, cell extracts were prepared and Western blotting was performed with specific anti-(PKC isoform) antibodies as described in the Materials and methods section. Results are from three independent experiments showing the same pattern of expression.

amounts of these PKC isoforms were not significantly altered by prolonged exposure to PMA.

Modulation of PKC activity

Many reports have demonstrated that PMA-mediated proliferative potentiation is associated with the activation of PKC

Table 1 PMA-mediated modulation of PKC activity

Cells were incubated with vehicle (control), 10 nM PMA or 10 μ M PMA, for 5 min, 30 min or 3 days. Subcellular fractions were then prepared and PKC kinase activity was measured in all three subcellular fractions as described in the Materials and methods section. Enzyme activity is expressed as pmol of 32 P incorporated/min per mg of protein, determined as described in the text. Results are means $\pm$ S.D. for six replicates from two independent experiments.

Treatment duration	Fraction	PKC activity (pmol/min per mg of protein)		
		Control	10 nM PMA	10 μ M PMA
5 min	Cytosol	172.1 $\pm$ 37.0	109.0 $\pm$ 25.8	44.5 $\pm$ 8.6
	Membrane	106.8 $\pm$ 16.2	168.0 $\pm$ 33.7	162.3 $\pm$ 31.2
	Nuclear	84.9 $\pm$ 20.8	84.6 $\pm$ 14.0	235.1 $\pm$ 24.0
30 min	Cytosol	213.6 $\pm$ 17.1	70.4 $\pm$ 23.5	38.9 $\pm$ 13.2
	Membrane	61.7 $\pm$ 12.4	149.1 $\pm$ 36.7	103.8 $\pm$ 17.7
	Nuclear	50.8 $\pm$ 9.6	188.6 $\pm$ 28.5	195.3 $\pm$ 15.9
3 days	Cytosol	135.7 $\pm$ 26.8	118.5 $\pm$ 35.0	23.0 $\pm$ 4.7
	Membrane	51.1 $\pm$ 22.0	93.9 $\pm$ 37.6	33.8 $\pm$ 3.3
	Nuclear	59.8 $\pm$ 15.2	98.0 $\pm$ 10.8	35.8 $\pm$ 6.8

activity. We measured the subcellular distribution of PKC activity after 5 min, 30 min and 3 days of exposure to PMA (Table 1). In the untreated control cells, PKC activity was detected mainly in the cytosolic fraction, whereas there was a rapid loss of PKC activity from the cytosolic fraction after exposure to 10 nM or 1 μ M PMA for 5 or 30 min. However, PKC activity was markedly increased in the membrane and nuclear fractions of PMA-treated cells. Prolonged (3 days) treatment with 1 μ M PMA caused drastic decreases in PKC activity in all three subcellular fractions compared with untreated control cells and cells treated with 10 nM PMA (Table 1). These results indicate that after severe depletion of cytosolic PKCs the membrane and nuclear PKCs are also depleted, after prolonged treatment with a high dose of PMA.

Re-distribution of PKC isoforms after treatment with PMA

Activation of PKCs by PMA is correlated with the translocation of PKC isoforms from cytosol to membrane or nucleus. In contrast, prolonged activation is correlated with PKC down-regulation. To determine whether PMA-mediated modulation of PKC activity was due to subcellular redistribution, cytosolic, membrane and nuclear fractions were prepared from controls and PMA-treated samples, then analysed by Western blotting (Figure 5) and immunocytochemical staining (Figure 6).

cPKCs

From the results of a Western blot analysis of untreated Hep3B cells, cPKC- α is present as a single 80 kDa protein mostly in cytosolic fraction; only small amounts of immunoreactivity were detected in the membrane and nuclear fractions (Figure 5). Treatment with 10 nM PMA caused the re-distribution of cPKC- α from the cytosol to the membrane and nuclear fractions within 5 min; the translocated cPKC- α remained in the nuclear fraction even at 2 h after PMA exposure. The immunoreactivities of cPKC- α returned to their normal cytosol-dominant pattern after 4 h incubation with 10 nM PMA. When exposed to 1 μ M PMA, cells exhibited a rapid and extensive translocation of cytosolic cPKC- α to the membrane and nucleus within 5 min; the immunoreactivity of cPKC- α was detected in small amounts in the membrane fraction and in large amounts in the nuclear fraction, and was almost completely abolished in the cytosolic fraction. This marked increase in nucleus-associated cPKC was transient

Figure 5 Western blot analysis of the PKC isoenzymes in subcellular fractions of PMA-treated Hep3B cells

Cells were treated as described in the legend to Figure 4. After incubation for the indicated durations (5 min, 30 min, 2 h, 8 h and 24 h) the subcellular fractions (C, cytosolic; M, membrane; N, nuclear) were obtained and analysed by Western blotting with specific anti-(PKC isoform) antibodies as described in the Materials and methods section. Results are from three independent experiments showing the same pattern of expression.

and maximal at 30 min incubation. However, the entire cellular pool (cytosolic, membrane and nuclear fractions) of cPKC- α was completely down-regulated after 8 h of incubation with 1 μ M PMA (Figure 5). Consistent with these immunoblotting results, immunofluorescence staining results indicated that cPKC- α (Figure 6, top panels) was distributed throughout the cytoplasm in untreated Hep3B cells. After 5 min of stimulation with PMA, cPKC- α was re-distributed to the cell periphery and accumulated in cell margins, including cell-cell junctions. Another portion of activated cPKC- α was concentrated in the perinuclear region; only small amounts were detected in intranuclear regions. After 30 min of treatment with PMA, cPKC- α was dominant in the perinuclear region, including the endoplasmic reticulum (Figure 6, top panels). However, cells treated with a high dose of PMA for up to 8 h showed a down-regulation of cPKC- α in all three subcellular fractions.

nPKCs and aPKCs

As shown in Figure 5, Figure 6 (middle panels) and Figure 6 (bottom panels), nPKC- δ and ϵ were present mainly in the membrane and nuclear fractions in untreated Hep3B cells. When exposed to PMA, the amounts of nPKC- δ and ϵ in the cytosolic and membrane fractions were decreased, whereas the nuclear amounts of these nPKCs increased after 5–30 min of exposure to PMA. Complete down-regulation of nPKC- δ was observed after 8 h of exposure to 1 μ M PMA. The subcellular distribution of nPKC- ϵ after 8 h of treatment with 1 μ M PMA was significantly down-regulated. Indirect immunofluorescence staining and Western blotting analysis revealed that nPKC- μ was localized mainly in the cytosol and the perinuclear region (Figure 7). aPKC- ζ was mostly found in the cytosol, with a small proportion detected in the membrane and nuclear fractions; aPKC- λ and ι were detected in all three fractions (Figure 7). In addition, aPKC- λ and aPKC- ι had similar subcellular localizations and immunocytochemical staining patterns. These results are in agreement with the results of previous reports that aPKC- ι is the human counterpart of mouse aPKC- λ [32,33]. Our experiments demonstrated that the total protein amounts and the subcellular

Figure 6 Immunocytochemical localization of PKC isoforms in PMA-treated Hep3B cells

Cells were plated on slides and processed for indirect immunofluorescence staining with specific anti-(PKC isoform) antibodies as described in the Materials and methods section. PKC immunostaining is shown in control, 10 nM PMA-treated and 1 μ M PMA-treated cells at 5 min, 30 min, 2 h and 8 h. Staining with anti-PKC α antibody (top panels); anti-PKC δ antibody (middle panels) and anti-PKC ϵ antibody (bottom panels). Cells were examined with a fluorescence microscope. Magnification $\times 270$. Results are from three independent experiments showing the same pattern of expression.

Figure 7 Subcellular localization of PKC- μ , ζ , ι and λ isoforms in Hep3B cells

Distributions of PKC- μ , ζ , ι and λ were analysed by Western blotting and indirect immunofluorescence staining with specific anti-(PKC- μ), anti-(PKC- ζ), anti-(PKC- ι) or anti-(PKC- λ) antibody. Results are from three independent experiments showing the same pattern of expression.

distributions of PKC- μ , ζ and λ/ι were not affected by PMA (results not shown).

DISCUSSION

Accumulating evidence indicates the involvement of PKC in modulating the proliferation of cells. Because cell proliferation potentiation and cellular responsiveness to diffusible signals change as a result of cell transformation [34], the application of tumour-promoting agents such as PMA to neoplastic cells might provide an insight into these changes as well as into the alterations of the cellular PKC isoforms. In this study we have explored the relationship between cell proliferative capacity and PKC activation in human hepatoma Hep3B cells. Results demonstrate that PMA induces a biphasic effect on the proliferation of Hep3B cells. A concentration of 10 nM PMA stimulated the growth of Hep3B cells but a higher concentration (1 μ M) impeded growth. Cell lines exhibiting various degrees of proliferative capacity demonstrate differential expressions of certain PKC isoforms [35,36]. Our biochemical and immunofluorescence studies showed that the ability of PMA to inhibit Hep3B cell growth is directly related to its capability of decreasing PKC activity and down-regulating PKC- α , δ and ϵ proteins. The results indicate that these three PKC isoenzymes have an important role in the regulation of Hep3B cell proliferation. However, the results presented here do not support the notion that the growth effect of PMA is associated with the modulation of a specific PKC isoform.

The present results also showed PMA-induced transient morphological changes in Hep3B cells. Both 10 nM and 1 μ M PMA-treated cells showed similar phenotypic alterations. Several studies have been conducted on the role of PKCs in the regulation of actin. The activation of PKC could affect the polymerization state and localization of actin. Hartwig et al. [37] indicated that a major intracellular PKC substrate, myristoylated alanine-rich C-kinase substrate (MARCKS), is an F-actin cross-linking protein. Phosphorylation of MARCKS by PKC might translocate MARCKS from membrane to cytosol and disassociate it from actin, followed by morphological alteration. Dwyer-Nield et al. [38] have demonstrated that PMA induces reversible morphological changes in lung epithelial cells when associated

with the activation of PKC. The activation of PKC- α caused actin rearrangement and phenotypic alteration [38], and calpain-dependent down-modulation of PKC- α was required for the reversible cytoskeletal changes [39]. Studies *in vitro* have demonstrated that PKC- α , δ and ϵ act as MARCKS protein kinases [39]. Ueberall et al. [40] reported that PKC- α and ϵ are responsible for MARCKS phosphorylation in intact NIH 3T3 fibroblasts. Our results are consistent with the results of previous reports. We found that the transient morphological changes in PMA-treated Hep3B cells were accompanied by the activation and down-modulation of PKC- α , δ and ϵ . Further study will be required to determine whether these PKCs are involved in the regulation of actin arrangement to mediate morphological changes in intact Hep3B cells.

From investigations of the expression and re-distribution of PKC isoenzymes, we found that the effect of PMA on PKC isoenzymes in Hep3B cells is limited to Ca^{2+} - and phospholipid-dependent isoenzymes, because the expressions of aPKC- ζ and ι/λ are unaffected by PMA. This result is consistent with the results of previous reports that demonstrated the lack of effect of PMA on aPKC- ζ and ι/λ isoenzymes in various cell types [1,3]. PKC- μ is also resistant to down-regulation by PMA. It has been noted that PKC- μ belongs to the novel/atypical PKCs, and has a low phorbol ester-binding capacity [41]. A previous study has demonstrated the lack of phorbol ester-induced down-regulation of PKC- μ *in vivo* [41]. Our present study showed rapid (5 min) and substantial increases in cPKC- α in membrane and nuclear fractions after exposure to PMA. In addition, this cPKC was retained in the nucleus for 0.5–2 h, then totally abolished after 4–8 h of incubation. Interestingly, the PKC- α isoform is expressed as a protein doublet after stimulation with 1 μ M PMA for 0.5–2 h. Preliminary studies have indicated that the upper band might be a phosphorylated and possibly more activated version of the lower band of this isoform. It will be of interest to analyse the critical role of PKC- α doublets in cellular response.

nPKC- δ and ϵ were found predominantly in the particulate fraction, were translocated from membrane to nucleus 15 min after stimulation with PMA, then down-regulated at 8 h. It is worth noting that the down-regulation of both conventional PKC- α and novel PKC (δ and ϵ) in Hep3B cells was detected only with 1 μ M PMA, not with 10 nM PMA. Similarly, in Swiss 3T3

cells, higher doses of PMA completely down-modulated PKC- α - δ and ϵ [42]. Furthermore our results do provide some evidence that in PMA-treated Hep3B cells increased PKC activity in nucleus reflects a translocation of PKC- α , δ and ϵ isoforms to this cellular compartment. The alterations in the nuclear PKC pool might initiate the transcription of genes after stimulation with a tumour-promoting agent. For many of the physiological effects in which PKC has a role, such as cell differentiation and mitogenesis, changes in nuclear function are involved. Many nuclear proteins, such as lamin B [43], histone H1 [44], RNA polymerase II [45], topoisomerase II [46], poly(ADP-ribose) synthetase [47], DNA methyltransferase [48] and DNA polymerase α [49], have been identified as possible substrates for phosphorylation by PKC. These observations and our present demonstration of a direct effect of PMA on human hepatoma nuclear PKC suggest that the nucleus is an important site for PKC-mediated growth control. It is interesting that focusing on nucleus-translocated PKCs enabled us to investigate nuclear events occurring during signal transduction. However, the exact cellular signals that result in the nuclear localization of PKC remain to be elucidated.

The results reported here indicate that the differential regulation of PKC isoenzymes, with respect to concentration and duration of exposure, represents different functions of PKC isoenzymes in the proliferation of Hep3B cells. Our findings suggest that certain PKC isoforms are activated and re-distributed in human liver cancer cells after treatment with a tumour-promoting agent. PKC expression and translocation have often been described as part of the pleiotrophic response to mitogens and tumour promoters [50]. In liver the translocation of PKC precedes the proliferative burst induced by partial hepatectomy [51,52]. Very little is known about the function of PKC in human liver cancer cells or the functional relationship of these PKC isoforms with proliferative potentiation. In this study, PMA-induced PKC isoenzyme modulation was well correlated with cell proliferative alterations in human hepatoma Hep3B cells. However, it remains to be determined which of the PKC isoform(s) in Hep3B cells might be directly involved in transient morphological changes and cell proliferative process.

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