Journal of Biomedical Science **12:** 289–296, 2005.
DOI 10.1007/s11373-005-1210-5

Activation of protein kinase C alpha is required for TPA-triggered ERK (MAPK) signaling and growth inhibition of human hepatoma cell HepG2

Wu Wen-Sheng* & Huang Jun-Ming
Department of Medical Technology, TZU CHI University, Taiwan

Received 25 May 2004; accepted in revised form 6 December 2004

Key words: extracellular related kinase, HepG2 cell, protein kinase C, tetradecanoyl phorbol acetate

Summary

The signaling mechanisms for most of the antiproliferative processes are not fully understood. We have demonstrated that ERK(MAPK) signaling was involved in the induction of both $p15^{INK4b}$and $p16^{INK4a}$ CDK inhibitors and growth inhibition of hepatoma cell HepG2 triggered by the tumor promoter tetradecanoyl phorbol acetate (TPA). In this study, the upstream signal mechanism for TPA-induced ERK(MAPK) activation was investigated. In HepG2 cells only one of the cPKC isozymes, PKCα, but not cPKCβII, nPKCε or aPKCζ was activated by TPA as demonstrated by its membrane translocation within 10–30 min and down-regulation at 24 h after TPA treatment. Pretreatment of 0.2–2.0 μM Bisindolylmaleimides, an inhibitor of PKC, attenuated the TPA-induced phosphorylation of ERK, gene expressions of $p15^{INK4b}$and $p16^{INK4a}$, and growth inhibition of HepG2 cell in a dose-dependent manner. Consistently, transfection of HepG2 with 1.0–3.0 μM antisense (AS) PKCα, but not (AS) PKCβII, or nPKCε oligonucleotides (ODN), for 36 h prior to TPA treatment also prevented the TPA-induced molecular and cellular effects described above. Taken together, we concluded that PKCα is specifically required for TPA-induced ERK(MAPK) signaling to trigger gene expressions of $p15^{INK4b}$and $p16^{INK4a}$ leading to HepG2 growth inhibition.

Introduction

Understanding the regulation of cell growth is fundamental for cancer research. The molecular mechanisms for growth inhibition of tumor cells exerted by a lot of anti-proliferative agents such as TGF-β, retinoic acid, Vitamin D3, phenylacetate, green tea, activin, tetradecanoyl phorbol acetate (TPA) and several Chinese herbs have been intensively studied in recent years [1–7]. In general, the induction of gene expressions of cyclin dependent kinase inhibitors (CDKIs), inhibition of CDK activity, and dephosphorylation of retinoblastoma (Rb) protein were associated with cell cycle arrest induced by these agents. However, the signaling mechanisms for most of these antiproliferative processes are not well understood except the Smad-dependent and independent pathways that mediate TGF-β-induced growth inhibition [for review, 8]. We previously reported the induction of both $p15^{INK4b}$and $p16^{INK4a}$ CDK inhibitors during growth inhibition of hepatoma cell HepG2 triggered by the Chinese herb Saikosaponin *a* and the tumor promoter TPA [9]. We further demonstrated that the extracellular related kinase (ERK), one of the mitogen activated protein kinases (MAPK), along with its down stream transcriptional system were associated with this antiproliferative process [7].

Protein kinase C (PKC) is the cellular receptor of TPA [10]. More than 12 PKC isozymes, the

*To whom correspondence should be addressed: Fax: +03-8571-917; E-mail: wuws@mail.tcu.edu.tw

conventional PKC, (cPKCα, βI, βII and γ), novel PKCs (nPKC-δ, -ε, -η) and atypical PKCs (aPKCζ) [for review, 11] have been found. The PKC isozymes presented tissue and cellular specific expressions as well as differences in cofactors and substrate dependence [12]. Previously, it was demonstrated that only three PKC isozymes (α, δ, ζ) were detected in rat liver homogenates [12]. Further, Tang et al. reported the expressions of cPKCα, βII, nPKCε and aPKCζ in rat hepatocyte [13]. The same expression patterns of PKC isozymes were also demonstrated in HepG2 cells [14]. Mounting evidence has indicated that various PKC isozymes are involved in cell cycle arrest [15–20]. For one example, PKCα activation was required for TPA-induced growth inhibition and $p21^{cip1}$ induction in HC11 mammary epithelial cells [18].

To understand the upstream signal mechanisms for TPA-induced ERK(MAPK) activation, the specificity of PKC isozymes for mediating the effects of TPA needs to be clarified.

Materials and methods

Cell culture and chemicals

HepG2 cells were cultured as described in our previous reports [7, 9]. TPA was purchased from Sigma (St. Louis, USA). Bisindolylmaleimides II and Manumycin A were purchased from Calbiochem (La Jolla, USA).

RT/PCR

RT/PCR for semi-quantitation of gene expression was performed as described in our previous report [7]. Briefly, HepG2 cells were seeded in 10-cm dishes for 48 h and treated as indicated. Total RNA was purified with Ultraspec RNA reagent (Biotecx, Houston, USA). First stranded cDNAs were synthesized by AMV transcriptase with oligo-dT primer from Invitrogen (Carlsbad, USA) using 1μg RNA of each sample. Semi-quantitation of the cDNA was performed by RT/PCR using the primer-dropping method as described in a previous report [21]. Each reverse transcript (i.e. $p15^{INK4b}$ and $p16^{ink4a}$ cDNA) was co-amplified with GAPDH for normalizing the amount of cDNA in each sample. The GAPDH primers were dropped at cycle 12 for $p15^{INK4b}$and $p16^{INK4a}$. The primers used were:

p15 ^{INK4b} F (5′→ATGCGCGAGGAGAACAA GGGCATG→3′)

R (5′→ TCAGTCCCCCGTGGCTGTGCGC AGGTA→3′) (413 bp)

p16 ^{INK4a} F (5′→CGGAAGGTCCCTCAGA CATC→3′)

R (5′→TCA TGA AGT CGA CAG CTT CCG →3′) (385 bp)

GAPDH F(5′→CGG AGT CAA CGG ATT TGG TCG TAT→3′)

R (5′→AGC CTT CTC CAT GGT GGT GAA GAC→3′) (306 bp)

PCR condition

After the initial denaturation at 95 °C for 5 min, amplification was performed with 3% formamide for 35 cycles of denaturation at 94 °C for 40 s, annealing at 53 °C for 1 min and extension at 72 °C for 40 s.

Quantitative analysis

For comparing the amount of PCR product between samples, a gel digitizing software, UN-SCAN-IT *gel* (version 5.1), was used for estimating the intensity of each band on the gel. Each experiment was repeated for 2–4-times. The coefficients of variation (CV) were approximately 4–8%.

Fractionation of cellular extract

Briefly, the cells were suspended in hypotonic buffer (10 mM Tris at pH 7.4, 50 mM NaCl, 0.3 mM Na-orthovanadate, 50 mM NaF, 1 mM DDT, 10 μg/ml leupeptin and 5 μg/ml aprotinin) and incubated for 30 min at 4 °C. The cell suspensions were homogenized and centrifuged at 2500 rpm for 3 min. The supernatants were then subjected to ultracentrifugation at 25,000 rpm for 1 h. The resulting supernatants were obtained as cytosolic fractions. The pellets were dissolved in lysis buffer containing 50 mM Tris at pH 7.4, 50 mM NaCl, 1% Triton X-100, 0.3 mM Na-orthovanadate, 50 mM NaF, 1 mM DDT, 10 μg/ml leupeptin and 5 μg/ml aprotinin. After centrifugation at 15,000 rpm for 30 min the supernatants were obtained as particulate fractions.

Western blotting

Western blotting was performed as described in our previous report [7]. Briefly, the cells were lyzed in buffer containing 50 mM Tris at pH 7.4, 50 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 0.3 mM Na-orthovanadate, 50 mM NaF, 1 mM DDT, 10 μg/ml leupeptin and 5 μg/ml aprotinin. An equal amount of protein in each sample was separated by 12% SDS/PAGE and transferred to PVDF membrane. Membranes were blocked in 20 mM Tris (pH 7.6) and 250 mM NaCl containing 5% dry milk and probed with antibodies against p-ERK-1, ERK-1, $p16^{INK4a}$, cPKCα, cPKCβII, nPKCε, aPKCζ (Santa Cruz, USA), $p15^{INK4b}$ (Oncogene, UK). Following incubation with alkaline phosphatase-conjugated goat anti mouse or goat anti-rabbit 2nd antibody, proteins were visualized with NBT/BCIP for color development. For quantitation, the blots were scanned and the intensity of each specific band was estimated using gel digitizing software UN-SCAN-IT *gel* version 5.1. Each experiment was repeated for 2–5-times. The CV were approximately 5.0–8.5%.

Transfection of antisense (AS) PKC oligodeoxynucleotide (ODN)

The sense (S) PKCα and antisense (AS) PKCα, βII, ε ODN with phosphorothioate linkage throughout the entire ODN molecule were the same as those in previous reports [22–24]: (AS) PKCα, 5′-CAT GGT YCC CCC CAA CCA CC-3′ Y = T or C (antisense sequence against 20 nucleotides upstream of the AUG codon), (S) PKCα,5′-GGT GGT TGG GGG GRA CCA TG-3′ R = A or G [22]; (AS) PKCβII 5′-CGC AGC CGG GTC AGC ATC-3′ (23); (AS) PKCε 5′-GCC ATT GAA CAC TAC CAT-3′ [24]. The PKC isozyme (ODNs) were transfected into HepG2 cells (at final concentration of 1.0–3.0 μM ODN) for 36 h using an effectene transfection kit from Gibco (Gaithersburg, USA) according to the manufacturer's instructions.

Results

It has been well established that translocation of PKC from cytosol to membrane is closely associated with its activation depending on Ca^{2+} and phosphatidylserine [25–27]. To investigate which of the PKC isotypes expressed in HepG2 (i.e. PKCα, βII, nPKCε and aPKCζ) are activated by TPA, Western blots of these PKC isozymes in both cytosolic and particulate fraction were performed. As demonstrated in Figure 1, PKCα was translocated from cytosol to particulate fraction after treatment with 50 nM TPA for 10–30 min followed by down-regulation at 24 h. Surprisingly, the calcium-independent nPKCε totally disappeared in the cytosol after 10 min of TPA treatment but did not accompany its increase in particulate fraction (in which a substantial amount of PKCε already existed). No cytosol-to-particulate translocation or down-regulation of cPKCβII and atypical PKCζ (zeta) was observed after TPA treatment (Figure 1). Taken together, these results suggest that PKCα is the only PKC isozyme activated on the membrane after TPA treatment in HepG2 cells.

To investigate whether activation of PKC is required for TPA-induced HepG2 growth inhibition, the cells were pretreated with Bisindolylmaleimides (abbreviated as *Bis* in the following text), a specific inhibitor of PKC, for 30 min to observe whether the effects of TPA were blocked. As shown in Figure 2a, HepG2 cell growth was inhibited by 48% after treatment with 50 nM TPA for 48 h. Pretreatment of 0.2–2.0 μM *Bis* for 30 min prevented the TPA-triggered inhibition of cell growth in a dose-dependent manner. In the sample pretreated with 2.0 μM *Bis*, almost 95% of the growth inhibitory effects of TPA were prevented. The results strongly suggested that the TPA-induced PKC activation is required for

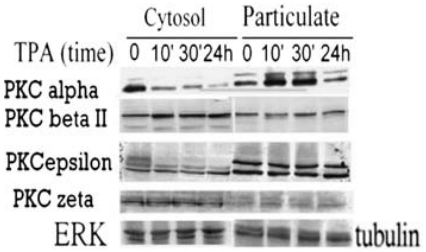

Figure 1. Subcellular distribution of PKC isozymes in HepG2 induced by TPA. HepG2 cells were treated with 50 nM TPA for 0, 10 min, 30 min and 24 h. Western blot of PKCα,PKCβII, PKCε and PKCζ in cytosol and particulate fraction of cell lysate at each time point were performed as described in Materials and methods. Western blot of ERK and tubulin were used as internal control for cytosolic and particulate fraction, respectively.

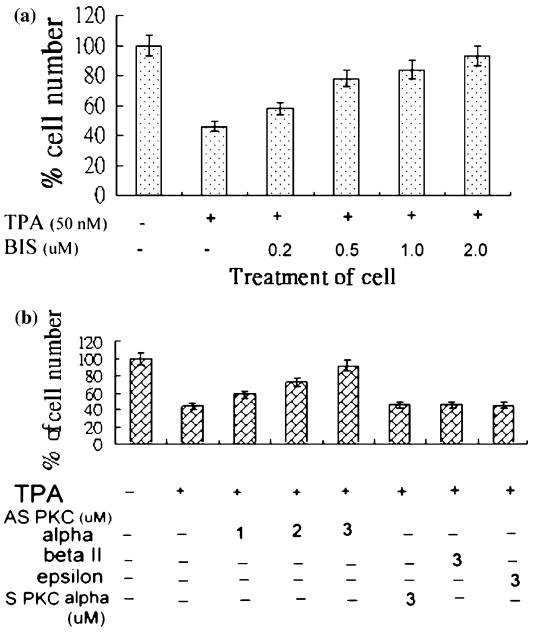

Figure 2. Prevention of TPA-induced HepG2 growth inhibition by bisindolylmaleimides and antisense (AS) PKCα (ODN). (a) HepG2 cells in 24 well plate (4×10^4/well) were untreated or pretreated with 0, 0.2–2.0 μM Bis for 30 min followed by treatment with 50 nM TPA for 48 h. (b) HepG2 cells in 24 well plate (4×10^4/well) were untreated or transfected with 0, 1.0, 2.0, 3.0 μM (AS) PKCα, 3.0 μM (S) PKCα, 3.0 μM (AS) PKCβII or 3.0 μM (AS) PKCε (ODNs) for 36 h followed by treatment with 50 nM TPA for 48 h. Cell numbers for each experimental group were counted using a hemocytometer. The cell numbers of the control group (without any treatment) was regarded as 100%. Each experiment was repeated for four times with a CV of 7.5%.

inhibiting HepG2 cell growth. To further investigate whether PKCα is the PKC isozyme involved, the antisense technique was used to observe whether blockage of PKCα translation influenced the effects of TPA. The cells were transfected with 1.0–3.0 μM (AS) PKCα (ODN) for 36 h before TPA treatment. As compared with the control HepG2 cells, the TPA-induced growth inhibitory effects were attenuated by 20–75% in HepG2 cells transfected with (AS) PKCα (ODN) (but not with (S) PKCα (ODN) as a negative control) in a dose-dependent manner (Figure 2b). In comparison, the TPA-triggered HepG2 growth inhibition was not prevented by (AS) PKCβII or (AS) PKCε (ODN) (Figure 2b). Western blots verified that translations of each PKC isozyme in cells transfected with its respective (AS) PKC (ODN) were indeed blocked. As shown in Figure 3a, about 95% reduction of the PKCα protein was observed in cells transfected with 3.0 μM (AS) PKCα but not in cells transfected with 3.0 μM (S) PKCα (ODN). Similarly, PKCβII and PKCε proteins were reduced by about 65 and 80% in cells transfected with 3.0 μM (AS) PKCβII and (AS) PKCε (ODN), respectively (Figure 3b).

To further investigate whether PKC activation was required for transmitting ERK(MAPK) signals, the cells were pretreated with *Bis* for 30 min followed by treatment with 50 nM TPA for 30 min. As shown in Figure 4a, the TPA-induced ERK phosphorylation reduced 50–70% using 0.5–2.0 μM *Bis* in a dose-dependent manner. Cells treated with *Bis* only did not show any changes of ERK phosphorylation. To investigate whether PKCα was the PKC isozyme responsible for ERK activation, the cells were transfected with (AS) PKCα (ODN) for 36 h prior to TPA treatment. As was expected, the TPA-induced ERK phosphorylation in cells transfected with 1.0–3.0 μM (AS) PKCα (ODN) were reduced by 20–70% in a dose-dependent manner (Figure 4b). In comparison, transfection of the cells with 3.0 μM (AS) PKCβII or PKCε (ODN) for 36 h did not show any preventative effects (Figure 4c).

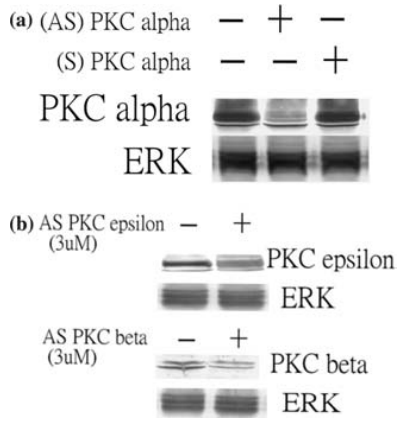

Figure 3. Reduction of PKCα protein by antisense (AS) PKCα (ODN). HepG2 cells were transfected with (a) 3.0 μM (AS) PKCα or (S) PKCα (ODN) and (b) 3.0 μM (AS) PKCβII or (AS) PKCε (ODN) for 36 h. "Control" represents the cells without any treatment. Western blot of PKCα, βII and ε were performed as described in Materials and methods. Western blot of ERK was performed for normalizing protein amount in each sample.

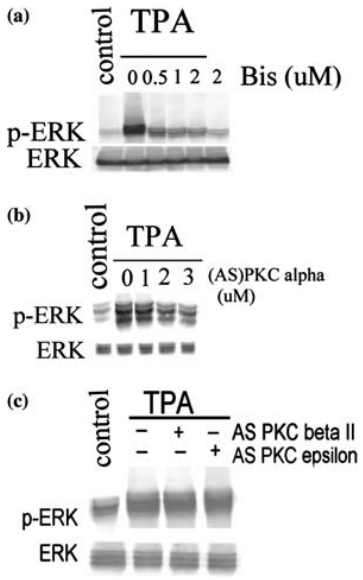

Figure 4. Blockage of TPA-induced ERK phosphorylation in HepG2 cell by Bisindolylmaleimides (*Bis*) and antisense PKC (ODNs). For the effect of *Bis*, HepG2 cells were untreated (control), treated with 2.0 μM *Bis* for 30 min or pretreated with 0, 0.5, 1.0, 2.0 μM *Bis* for 30 min followed by treatment with 50 nM TPA for 30 min (a). For the effect of the antisense (AS) PKC isozymes, HepG2 cells were transfected with (b) 0,1.0, 2.0 or 3.0 μM (AS) PKCα (ODN) and (c) 3.0 μM (AS) PKCβII or PKCε (ODN) for 36 h followed by treatment with 50 nM TPA for 30 min. "Control" represents the cells without any treatment. Western blot of phosphorylated ERK in each experimental group was performed as described in Materials and methods. Western blot of ERK (bottom panel) was used for normalizing the protein amount.

Since we demonstrated that TPA-induced $p15^{INK4b}$and $p16^{INK4a}$ gene expressions were mediated by ERK(MAPK) signaling [7], we further investigated whether they were also mediated by PKC activation. As demonstrated by RT/PCR in Figure 5a, expressions of both $p15^{INK4b}$ and $p16^{INK4a}$ genes were greatly induced by TPA at 24 h. Pretreatment of 0.5 and 1.0 μM *Bis* for 30 min dramatically suppressed the TPA-induced expression of $p15^{INK4b}$ and $p16^{INK4a}$ genes by 80–90% and 60–90%, respectively. Treatment of the cells with 1.0 μM *Bis* only did not influence the expression of the genes, consistently, Western blot of $p15^{INK4b}$and $p16^{INK4a}$ demonstrated that pretreatment of 1.0 μM *Bis* blocked the TPA-induced $p15^{INK4b}$and $p16^{INK4a}$ proteins by 95 and 75%, respectively (Figure 5d). Furthermore, transfection of the cells with 3.0 μM (AS) PKCα (ODN) for 36 h prior to TPA treatment also suppressed the TPA-induced $p15^{INK4b}$and $p16^{INK4a}$ gene expressions by 95 and 85%, respectively, as shown by RT/PCR in Figure 5b. In comparison, the TPA-induced $p15^{INK4b}$and $p16^{INK4a}$ gene expressions were not prevented by (AS) PKCε or (AS) PKCβII (ODN) (Figure 5c). In addition, Western blot analysis (Figure 5e) demonstrated $p15^{INK4b}$ protein induced by TPA was almost prevented by (AS) PKCα (ODN) confirming the RT/PCR data. Taken together, the results suggest that PKCα isozyme specifically mediated ERK(MAPK) signaling for TPA-induced gene expressions of $p15^{INK4b}$ and $p16^{INK4a}$ that ultimately led to growth inhibition of HepG2.

Discussion

One of the mechanisms of uncontrolled cellular proliferation is escape of cells from normal growth regulatory machinery such as that operated by transforming growth factor β (TGF-β)[28–29]. Understanding the detailed molecular mechanisms for growth inhibitions of tumor cells exerted by anti proliferative agents might help researchers in developing chemotherapy and gene therapy to treat cancer patients. The ERK(MAPK) signaling pathway is known to be associated with cell cycle arrest in tumor cells including MCF-7 breast cancer cells [30–31], prostate cancer cells [32], colorectal cancer cells [33], astrocytic tumor cells [34] and small cell lung cancer [35]. However, few of the researchers used antiproliferative agents as tools for comprehensively examining the ERK (MAPK) signal pathways leading to growth inhibition of tumor cells. In this report, we used TPA triggered-HepG2 growth inhibition as a model to address this issue. Our results demonstrated that TPA-induced activation of ERK(MAPK), gene expression of $p15^{INK4b}$ and $p16^{INK4a}$, and HepG2 growth inhibition were mediated by PKCα in a Ras-independent manner. Among the PKC isozymes expressed in HepG2, PKCα was the only one that exhibited cytosol-to-particulate translocation within 10–30 min and down-regulated after 24 h (Figure 1). The results suggest that PKCα is the key PKC isozyme responsible for transmitting the TPA triggered antiproliferative signals. Although down-regulation of cytosolic PKCε was observed, whether this event was associated with

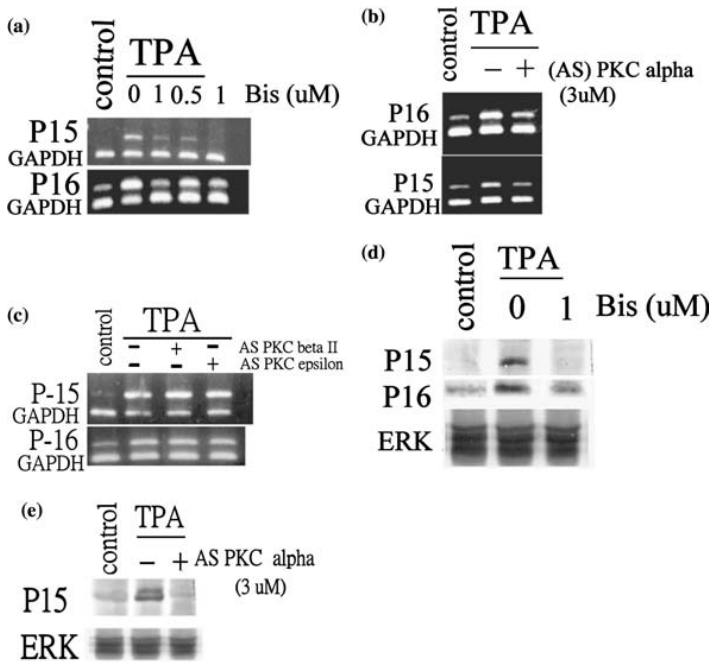

Figure 5. Prevention of TPA-induced $p15^{INK4b}$ and $p16^{INK4a}$ gene expression by *Bis* and (AS) PKCα but not (AS) PKC*β*II or ε (ODN). For RNA analysis, HepG2 cells were pretreated with *Bis* at indicated concentration for 30 min (a) or transfected with none or 3.0 μM (AS) PKCα (ODN) (b) and with none or 3.0 μM (AS) PKC*β*II or ε (ODN) (c) for 36 h, followed by treatment with 50 nM TPA for 24 h. The last lane in (a) represents the cells treated with 1.0 μM *Bis* only. "Control" represents the cells without any treatment. RT/PCR of $p15^{INK4b}$ and $p16^{INK4a}$ were performed as described in Materials and methods. GAPDH was co-amplified as an internal control. For protein analysis, HepG2 cells were pretreated with *Bis* at indicated concentration for 30 min (d) or transfected with none or 3.0 μM (AS) PKCα (ODN) for 36 h (e), followed by treatment with 50 nM TPA for 24 h. "Control" represents the cells without any treatment. Western blot of $p15^{INK4b}$ was performed as described in Materials and methods, ERK was used for normalizing the protein amount.

the effects of TPA on HepG2 cell growth is not clear. Moreover, the specificity of PKCα was confirmed when (AS) PKCα (ODN) specifically prevented the HepG2 growth inhibition, ERK activation and $p15^{INK4b}$/$p16^{INK4a}$ gene expressions induced by TPA whereas (AS) PKC*β*II and PKCε (ODN) did not (Figures 2–5).

Although PKC is known to activate Ras-Raf-MEK-MAPK cascade, the role of Ras for PKC-mediated MAPK activation seems ambiguous. In some reports, Ras may cooperate with PKC or activated by PKC to transmit the Raf/MAPK signal [36–40]. In the others, PKC may bypass Ras and activate ERK(MAPK) through interaction with Raf or MEK [41–44]. We have performed the Ras activation assay and found that Ras activity was in fact slightly reduced by TPA (data not shown) implying that Ras is not involved in PKC-mediated ERK signaling in HepG2. Thus, PKC may directly induce Raf or MEK, the downstream effector molecules of Ras, to trigger ERK signaling in HepG2. Further studies using inhibitors and mutants of Raf and MEK are needed to address this issue.

It is interesting to note that PKC-mediated ERK(MAPK) kinase activation can be associated not only with growth inhibition (as in our study) but also with cell proliferation [45–47] and differentiation [42]. This implies that one signaling pathway may trigger different gene expression profiles for various cellular processes. Identification of which factors induced by the PKC–ERK (MAPK) pathway are responsible for determining the ultimate fate of a cell is worthy of further investigation.

Acknowledgements

We would like to thank the National Science Council (NSC) in Taiwan for financial support and Mr. Chung for technical assistance.

References

1. Weber E., Ravi R.K., Knudsen E.S., William J.R., Dillehay L.E., Nelkin B.D., Kalemkerian G.P., Feremisco J.R. and Mabry M., Retinoic acid-mediated growth inhibition of small cell lung cancer cells is associated with reduced myc and increased p27Kip1 expression. Int. J. Cancer 80(6): 935–943, 1999.
2. Verlinden L., Verstuyf A., Van Camp M., Marcelis S., Sabbe K., Zhao X.Y., De Clercq P., Vandewalle M. and Bouillon R., Two novel 14-Epi- analogues of 1,25-dihydroxyvitamin D3 inhibit the growth of human breast cancer cells *in vitro* and *in vivo*. Cancer Res. 60(10): 2673–2679, 2000.
3. Kavanagh K.T., Hafer L.J., Kim D.W., Mann K.K., Sherr D.H., Rogers A.E. and Sonenshein G.E., Green tea extracts decrease carcinogen induced mammary tumor burden in rats and rate of breast cancer proliferation in culture. J. Cell. Biochem. 82(3): 387–398, 2001.
4. Onishi T., Yamakawa K., Franco O.E., Suzuki R. and Kawamura J., P27 kip1 is the key mediator of phenylacetate induced cell cycle arrest in human prostate cancer cells. Anticancer Res. 20(5A): 3075–3081, 2000.
5. Robson C.N., Gnanapragasam V., Byrne R.L., Collins A.T. and Neal D.E., Transforming growth factor-beta1 up-regulates p15, p21 and p27 and blocks cell cycling in G1 in human prostate epithelium. J. Endocrinol. 160: 257–266, 1999.
6. Zauberman K., Oren M. and Zipori D., Involvement of p21(WAF1/Cip1), CDK4 and Rb in activin A mediated signaling leading to hepatoma cell growth inhibition. Oncogene 15: 1705–1711, 1997.
7. Wu W.S., ERK signalling is involved in p15/p16 expression and HepG2 growth inhibition triggered by TPA and Saikosaponin a. Oncogene 22: 955–963, 2003.
8. Derynck R. and Zhang Y.E., Smad-dependent and Smad-independent pathways in TGF-β family signaling. Nature 425(9): 577–584, 2003.
9. Wu W.S. and Heu H.Y., Involvement of $p15^{INK4b}$ and $p16^{INK4a}$ gene expression in Saikosaponin a and TPA induced growth inhibition of HepG2 cells. Biochem. Biophys. Res. Commun. 285: 183–187, 2001.
10. Burns D.J. and Bell R.M., Protein kinase C contains two phorbol ester binding domain. J. Biol. Chem. 266(27): 18330–18338, 1991.
11. Dekker L.V., McIntyre P. and Parker P.J., Protein knase C a question of specificity. Trends. Biochem. Sci. 19: 73–77, 1994.
12. Wetsel W.C., Khan W.A., Merchenthaler I., Rivera H., Halpern A.E., Phung H.M., Negro-Vilar A. and Hannun Y.A., Tissue and cellular distribution of the extended family of protein kinase C isoenzymes. J. Cell. Biol. 117(1): 121–133, 1992.
13. Tang E.Y., Parker P.J., Beattie J. and Houslay M.D., Diabetes induces selective alterations in the expression of protein kinase C isoforms in hepatocytes. FEBS Lett. 326(1–3): 117–123, 1993.
14. Ducher L., Croquet F., Gil S., Davy J., Feger J. and Brehier A., Differential expression of five protein kinase C isoenzymes in FAO and HepG2 hepatoma cell lines compared with normal rat hepatocytes. Biochem. Biophys. Res. Commun. 14: 217(2): 546–553, 1995.
15. Mauro A., Cicarelli C., De Cesaris P., Scoglio A., Bouche M., Molinnaro M., Aquino A. and Zani B.M., PKC alpha-mediated ERK, JNK and p38 activation regulates the myogenic program in human rhabdmyosarcoma cells. J. Cell. Sci. 115(pt 18): 3587–3599, 2002.
16. Tibudan S.S., Wang Y. and Denning M.F., Activation of protein kinase C triggers irreversible cell cycle withdrawal in human keratinocytes. J. Invest. Dermatol. 119(6): 1282–1289, 2002.
17. Detjen K.M., Brembeck F.H., Welzel M., Kaiser A., Haller H., Wiedenmann B. and Rosewicz S., Activation of protein kinase C alpha inhibits growth of pancreatic cancer cells via p21 (cip)-mediated cell cycle arrest. J. Cell. Sci. 113(pt 17): 3025–3035, 2000.
18. Slosberg E.D., Klein M.G., Yao Y., Han E. K-H., Schieren I. and Weistein I.B., The α isoform of protein kinase C mediate phorbol ester- induced growth inhibition and p21cip induction in HC11 mammary epithelial cells. Oncogene 18(48): 6658–6666, 1999.
19. Kosa K., Rosenberg M.I., Chiantore M.V. and De Luca L.M., TPA induces transglutaminase C and inhibit cell growth in the colon carcinoma cell line SW620. Biochem. Biophys. Res. Commun. 232(3): 737–741, 1997.
20. Tchou W.W., Rom W.N. and Tchou-Wong K.M., Novel form of p21 (WAF/CIP1/SDI1) protein in phorbol ester-induced G2M arrest. J. Biol. Chem. 371(47): 29556–29560, 1996.
21. Wong H., Anderson W.D., Cheng T. and Riabowol K.T., Monitoring mRNA expression by polymerase chain reaction: the ''primer-dropping'' method. Anal. Biochem. 223(2): 251–258, 1994.
22. Buitrago C.G., Pardo V.G., de Boland A.R. and Boland R., Activation of Raf-1 through Ras and PKCα mediates 1α, 25$(OH)_2$-vitamin D3 regulation of the MAPK pathway in muscle cell. J. Biol. Chem. 278(4): 2199–21205, 2003.
23. Senthil K.V., Sridevi D., Teddy Y. and Ishwarlal J., α-Tocopherol decreases superoxide anion release in human monocytes under hyperglycemic conditions via inhibition of protein kinase C-α. Diabetes 51: 3049–3054, 2002.
24. Uemura K., Aki T., Yamaguchi K. and Yoshida K., Protein kinase C-epsilon protects PC12 cells against methamphetamine-induced death: possible involvement of suppression of glutamate receptor. Life Sci. 72(14): 1595–1607, 2003.
25. Keranen L.M. and Newton A.C., Ca^{2+} differentially regulates conventional protein kinase Cs' membrane interaction and activation. J. Biol. Chem. 272(41): 25959–25967, 1997.
26. Oancea E. and Meyer T., Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. Cell. 95(3): 307–318, 1998.
27. Bolsover S.R., Gomez-Fernandez J.C. and Corbalan-Garcia S., Role of the Ca^{2+}/phosphatidylserine binding region of the C2 Domain in the translocation of protein kinase Cα to the plasma membrane. J. Biol. Chem. 278(12): 10282–10290, 2003.

28. Gold L.I., The role for transforming growth factor beta in human cancer. Crit. Rev. Oncogenesis 14(4): 303–360, 1999.
29. Derynck R., Akhurst R.J. and Balmain A., TGFβ signaling in tumor suppression and cancer progression. Nat. genet. 29: 117–129, 2001.
30. Sato H., Ogata H. and De Luca L.M., Annexin V inhibit the TPA induced activation of Ras/ERK signalling pathway upstream of Shc in MCF-7 cells. Oncogene 19(25): 2904–2912, 2000.
31. Albas J., Rivka S.-D., Steenberg P.H. and Sussenbach van der Burg B., The role of MAP kinase in TPA mediated cell cycle arrest of human breast cancer cells. Oncogene 16: 131–139, 1998.
32. Ravi R.K., McMahon M., Yanggang Z., Williams J.R., Dillehay L., Nelkin B.D. and Mabry M., Raf-1 induced cell cycle arrest in LNcap human prostate cancer cells. J. Cell. Biochem. 72(4): 458–469, 1999.
33. Park K.S., Ahn Y., Kim J.A., Yun M.S., Seong B.L. and Choi K.Y., Extracellular Zin stimulate ERK dependent activation of p21(Cip/WAF1) and inhibit proliferation of colorectal cancer cells. Br. J. Pharmacol. 137(5): 597–607, 2002.
34. Fanton CP., McMahon M. and Pieper R.O., Dual growth arrest pathways in astrocytes and astrocytic tumors in response to Raf-1 activation. J. Biol. Chem. 276(22): 18871–18877, 2001.
35. Ravi R.K., Weber E., McMahon M., Williams J.R., Baylin S., Mal A., Harter M.L., Dillehay L.E., Claudio P.P., Giordano A., Nelkin B.D. and Mabry M., Activated Raf-1 causes growth arrest in human small cell lung cancer cells. J. Clin. Invest. 101(1): 153–159, 1998.
36. Liu J.F., Crepin M., Liu J.M., Barritault D. and Ledoux D., FGF-2 and TPA induce matrix metalloproteinase-9 secretion in MCF-7 cells through PKC activation of the Ras/ERK pathway. Biochem. Biophys. Res. Commun. 293(4): 1174–1182, 2002.
37. Katagiri K., Hattori S., Nakamura S., Yamamoto T., Yoshida T. and Katagiri T., Activation of Ras and formation of GAP complex during TPA-induced monocytic differentiation of HL-60 cells. Blood 84(6): 1780–1789, 1994.
38. Thomas S.M., DeMarco M., D'Arcangelo G., Halegoua S. and Brugge J.S., Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. Cell 68(6): 1031–1040, 1992.
39. Alexandropoulos K., Qureshi S.A. and Foster D.A., Ha-Ras functions downstream from protein kinase C in v-Fps-induced gene expression mediated by TPA response elements. Oncogene 8(3): 803–807, 1993.
40. Hess A., Wijayanti N., Neuschafer-Rube A.P., Katz N., Kietzmann T. and Immenschuh S., Phorbol ester-dependent activation of peroxiredoxin I gene expression via a protein kinase C, Ras, p38 mitogen-activated protein kinase signaling pathway. J. Biol. Chem. 278(46): 45419–45434, 2003.
41. Ueda Y., Hirai S., Osada S., Suzuki A., Mizuno K. and Ohno S., Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. J. Biol. Chem 271(38): 23512–23519, 1996.
42. Soderholm H., Olsson A., Lavenius E., Ronnstrand L. and Nanberg E., Activation of Ras, Raf-1 and protein kinase C in differentiating human neuroblastoma cells after treatment with phorbol ester and NGF. Cell. Signal. 13(2): 95–104, 2001.
43. Tobin D., Nilsson M. and Toftgard R., Ras-independent activation of Rel-family transcription factors by UVB and TPA in cultured keratinocytes. Oncogene 12(4): 785–793, 1996.
44. Barnard D., Diaz B., Clawson D. and Marshall M., Oncogenes, growth factors and phorbol esters regulate Raf-1 through common mechanisms. Oncogene 17(12): 1539–1547, 1998.
45. Iijima Y., Laser M., Shiraishi H., Willey C.D., Sundaravadivel B., Xu L., McDermott P.J. and Kuppuswamy D., c-Raf/MEK/ERK pathway controls protein kinase C-mediated p70S6K activation in adult cardiac muscle cells. J. Biol. Chem. 277(25): 23065–23075, 2002.
46. Dorothee C.S., Richard M.M., Chritopher J., Marshall and Peter J.P., Activation of the mitogen-activated protein kinase/extracellular signal-related kinase pathway by conventional, novel and atypical protein kinase C Isotypes. Mol. Cell. Biol. 18(2): 790–798, 1998.
47. Yang C.M., Chien C.S., Ma Y.H., Hsiao L.D., Lin C.H. and Wu C., Bradykinin B2 receptor-mediated proliferation via activation of the Ras/Raf/MEK/MAPK pathway in rat vascular smooth muscle cells. J. Biomed. Sci. 10(2): 208–218, 2003.