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Phorbol 12-myristate 13-acetate inhibits FRO anaplastic human thyroid cancer cell proliferation by inducing cell cycle arrest in G1/S phase: Evidence for an effect mediated by PKC δ .

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Running title: Antiproliferative effects of PMA in FRO cells.

Abstract

Phorbol 12-myristate 13-acetate (PMA) is known to affect a variety of cellular processes, including cell proliferation, differentiation, and migration. PMA has been shown to promote antiproliferative and antimigratory effects in many types of cancer cells. Our findings show that PMA induced a strong antiproliferative effect in two anaplastic (FRO and ARO) and one follicular (ML-1) thyroid cancer cell lines, and increased the fraction of FRO cells in G1 phase of the cell cycle. The fractions in the S- and G2 phases were decreased. Moreover, PMA evoked a significant increase in the levels of the cell cycle regulators p21^{Waf1/Cip1} and p27^{Kip1}. The levels of cyclin D3 and the cyclin dependent kinases cdk4 and cdk6 decreased, as did the phosphorylation of the Rb-protein. PMA did not induce apoptosis. PMA stimulated the translocation of protein kinase C (PKC) α , β I and δ isoforms to the cell membrane. PKC δ small interfering RNA attenuated the PMA-induced antiproliferative effect and prevented the upregulation of p21^{Waf1/Cip1} and p27^{Kip1}. Prolonged stimulation with PMA decreased the phosphorylation of mitogen-activated protein (MAP) kinase. PMA also decreased the phosphorylation of Akt and evoked a biphasic change in the phosphorylation of the forkhead box class-O protein (FOXO): an increase in phosphorylation, followed by a dephosphorylation. In addition, PMA inhibited FRO, ARO and ML-1 cell migration toward serum. The inactive phorbol ester analog 4 α -phorbol and the diacylglycerol analog 1,2-dioctanoyl-sn-glycerol were without an effect on proliferation and migration. The results indicate that PMA is an effective inhibitor of thyroid cancer cell proliferation and migration by a mechanism involving PKC-MAP kinase/Akt and FOXO signaling.

46

Key words: thyroid, cancer, phorbol esters, cell cycle, protein kinase C.

48

49 Introduction

50 Protein kinase C (PKC) is a family of serine-threonine kinases that regulate many
51 cellular processes including proliferation and survival. The numerous isotypes of PKC exert
52 important functions in the regulation of the cell cycle and intracellular signal transduction
53 (Nishizuka, 1986; Levesque and Siard, 1995). PKC-mediated signal transduction pathways
54 convert extracellular stimulation into a variety of cellular functions (Griner and Kazanietz,
55 2007). Phorbol 12-myristate 13-acetate (PMA) is a natural compound originally isolated from
56 the seed oil of the plant *Croton tiglium* and is a well known activator of PKC (Griner and
57 Kazanietz, 2007). The effect of PMA varies depending on cell type, the relative expression
58 and distribution of different phorbol ester receptors and their downstream effectors
59 (Nakagawa et al., 2005). In most normal cell types investigated, activation of PKC by PMA
60 enhances proliferation, while the proliferation of different cancer cell lines is inhibited upon
61 stimulation with PMA (Griner and Kazanietz, 2007).

62 Activation of PKC by PMA involves acute translocation of the PKC isoforms (PKCs) to
63 the plasma membrane, which occurs within a few minutes after stimulation (Newton, 2001).
64 The endogenous analogue of phorbol esters is diacylglycerol (DAG). Although the activation
65 of PKC by DAG is a transient event due to its fast metabolism, the action of phorbol esters is
66 sustained, leading to prolonged activation of PKCs (Blumberg et al., 1983).

67 The regulation of proliferation is a complicated process and is regulated by different
68 cyclin-dependent kinases (cdks), which are activated by binding to different types of cyclin
69 forming cyclin/cdk complexes to control the cell cycle (Damia and Broggin, 2004). During
70 the G1 phase the cyclin-dependent kinase inhibitors p21^{Waf1/Cip1} and p27^{Kip1} negatively
71 regulate the activity of the cyclin/cdk complexes (Sinibaldi et al., 2000). Another critical
72 effector in the G1 phase is the retinoblastoma (Rb)-protein (Dyson, 1998, Nevins, 1998).

The phosphoinositide-3 kinase (PI-3 kinase)/Akt pathway is also an important regulator of proliferation and has been implicated in thyroid tumorigenesis and particularly in the inactivation of p21^{Waf1/Cip1} (Liang and Singerland 2003; Shinohara et al., 2007). Furthermore the forkhead box class-O (FOXO) transcription factors play critical roles in a variety of physiological and pathological processes including cancer and are subject to negative regulation by the PI-3 kinase/Akt signal transduction pathway (Arden, 2006; Myatt and Lam, 2007). FOXO can also upregulate p27 and p21 gene expression (Medema et al., 2000; Dehan and Pagano, 2005).

PKC is involved in the control of the mitogen-activated protein (MAP) kinase pathway, which is responsible for controlling of diverse cellular responses, such as proliferation, differentiation and cell death (Pettersson et al., 2004). In NPA thyroid cancer cells, PKC δ activation has been shown to induce growth arrest by a mechanism dependent on MAP kinase (Koike et al., 2006). Recently we have shown that the MAP kinase pathway is an important component in the regulation of thyroid cancer cell proliferation (Afrasiabi et al., 2007) and in our previous studies we were able to show some implications for the role of PKC in thyroid cancer cell signaling (Afrasiabi et al., 2006). In addition to regulating proliferation, cell migration is another important biological process which is regulated by PKC (Nishizuka 1988). While PKC δ inhibition has been shown to limit the migration of prostate cancer cells (Kharait et al., 2006), PMA has been indicated as a negative regulator of breast cancer cells migration by targeting PKC α (Gauthier et al., 2003).

Previous studies have shown that by activation of PKC in dog thyroid cells, or in rat FRTL-5 thyroid cells, PMA promotes cell proliferation (Roger et al., 1986; Takada et al., 1988; Lombardi et al., 1988). In human cells in culture, a biphasic effect is observed: in short-term cultures, PMA inhibits proliferation, but in cells grown for longer periods, PMA

stimulates proliferation (Kraiem et al., 1995). However, in thyroid cancer cells, activation of PKC with PMA seems to have an inhibitory effect on proliferation (Koike et al., 2006).

The aim of the present study is to clarify the role of PKC in the growth and survival of anaplastic thyroid FRO cancer cells. We thus investigated the effect of PMA on proliferation and the cell cycle regulatory proteins, and on cell migration. Our investigation revealed that PMA is a potent inhibitor of both proliferation and migration in thyroid cancer cells.

Materials and methods

Materials. RPMI 1640 medium (without L-glutamine) and non-essential aminoacids were from Cambrex (Verviers, Belgium). L-glutamine, foetal bovine serum (FBS), penicillin and streptomycin were from GIBCO (Grand Island, NY, USA). DMEM (Dulbecco's modified Eagle's medium), phorbol 12-myristate 13-acetate (PMA), 4 α -phorbol, DOG and propidium iodide were from Sigma (St.Louis, MO, USA). GF 109203X, calphostin C, rottlerin, PD 98059, UO 126, LY 294002 and GÖ 6976, ALLM and ALLN were from Calbiochem (Darmstadt, Germany). Celltiter 96 solution cell proliferation assay was purchased from Promega corporation (Madison, WI, USA). Human Collagen type I was from Becton Dickinson Biosciences (Bedford, MA, USA). Primary antibodies against p21^{waf1/cip1}, p27^{kip1}, cyclin D1, cyclin D2, cyclin D3, cyclin E, cdk2, cdk4, cdk6, Rb, phospho-Rb (Ser-780), phospho-Rb (Ser-795), p44/42 MAP kinase, phospho-p44/42 MAP kinase (Thr-202/Tyr-204), Akt, phospho-Akt (Ser-473), phospho-Akt (Thr-308), FoxO1, phospho-FoxO1 (Ser-256), phospho-FoxO1 (Thr-24) and β -actin were obtained from Cell Signaling Technology (Danvers, MA, USA). Isoenzyme-specific PKC antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PKC β I specific inhibitory peptide (KIBI31-1) was obtained from KAI Pharmaceuticals (South San Francisco, CA, USA). The secondary

antibodies used were horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies (Sigma). Culture dishes were purchased from Falcon Plastics (Becton, NJ, USA).

Cell culture. The FRO and ARO human anaplastic thyroid cancer cell lines were a generous gift from Dr. James Fagin (Memorial Sloan-Kettering Cancer Center, NY, USA). The ML-1 follicular thyroid cancer cells were provided generously by Dr. Johann Schönberger (University of Rosenberg, Germany). FRO and ARO cells were cultured in RPMI 1640 medium and ML-1 cells were cultured in DMEM medium supplemented with 10% FBS, 1% non-essential aminoacids, 2 mM L- glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin and were grown in a water-saturated atmosphere containing 5% CO₂ and 95% air at 37 °C. The day before an experiment the medium was removed and replaced with RPMI containing 5% dextran-treated-charcoal-stripped FBS.

³H-thymidine incorporation assay. Cell proliferation was determined using ³H-thymidine incorporation (Amersham, Buckinghamshire, UK). For the assay 50 000 cells were cultured for 48 h on 35-mm plates in a water-saturated atmosphere containing 5% CO₂ and 95% air at 37 °C followed by 24 h of treatment with the indicated concentrations of PMA. At the day of the experiment, the cultures were pulsed with ³H-thymidine (0.4 µCi/ml) for 4 h of incubation. Cells were washed three times with ice cold PBS followed by a 10 min incubation with 5% perchloric acid and another 10 min with 0.1 N NaOH. Cells were harvested and radioactivity was counted using a Wallac 1410 liquid scintillation counter (Wallac OY, Turku, Finland). ³H-thymidine uptake was expressed as mean counts per minute (cpm) of triplicate samples. The results were confirmed by cell counting using trypan blue.

Celltiter assay. The cells (10 000 cells/well), seeded into 96-well plates for 24 h, were incubated with different concentrations of PMA for 1-20 h at 37 °C in 5% CO₂ and 95% air. After adding 100 µl/well of celltiter 96 reagent solution (5mg/ml), the plates were incubated for another 2 h. Optical density was determined at 540 nm using the Viktor fluorescence analyser (PerkinElmer Life Sciences, Turku, Finland).

Cell migration assay. Migration experiments were performed on 6.5 mm-diameter Transwell (Corning Costar, Bodenheim, Germany) chambers with 8 µM pore size. The filters were coated with collagen I (5µg/cm²) and then placed into the lower chamber. Lipid-stripped FBS was placed in the lower chamber as a chemoattractant. Cells were harvested and diluted with RPMI medium containing 0.1% BSA, 100 000 cells were added to the upper chamber and treated as indicated. The chambers were incubated in a humidified incubator at 37 °C in 5% CO₂/ 95% air for 6 h. The cells that traversed the filter and spread on the lower surface of the filter were fixed with 2% paraformaldehyde in PBS for 10 min, and stained with 0.1% crystal violet in 20% methanol for 5 min. The membranes were rinsed and allowed to dry. Nonmigratory cells on the upper membrane surface were removed with a cotton swab. The number of migratory cells/membrane was counted with 40x magnification in eight microscopic fields in a straight line bisecting the membrane.

Western blot analysis. FRO cells were cultured in 5% lipid stripped FBS medium for 1 day and stimulated with 100 nM PMA for the indicated time. After stimulation, FRO cells were washed 3 times with ice-cold PBS and lysed in cell lysis buffer [10mM Tris (pH 7.7), 150 mM NaCl, 7 mM EDTA, 0.5% NP-40, 0.2 mM PMSF (phenylmethylsulfonyl fluoride) and 0.5 µg/ml leupeptin], the lysate was centrifuged at 13 000 rpm for 15 min at +4 °C and the supernatant was collected. The protein concentration was determined using the BCATM Protein

Assay Kit (Pierce, Rockford, IL, USA). The samples were then stored at -20°C . Samples (15 μg of protein/sample) were subjected to SDS/PAGE (10% polyacrylamide). The proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by wet-blotting. Western blot analysis was performed using specific antibodies to the indicated proteins. The secondary antibodies used were horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies. The proteins were detected by enhanced chemiluminescence. For loading controls, membranes were washed with 1M Tris (pH 7.6), stripped for 20 min in 0.1 M glycine (pH 2.5) and subjected to anti β -actin antibody. Densitometric analysis was performed using ImageJ program for image analysis (NIH, Bethesda, MD, USA) and results were corrected for protein loading by normalization for β -actin expression.

Western blot of PKC isoenzymes. Cells were harvested in Hepes-buffered salt solution (HBSS; 118 mM NaCl, 4.6 mM KCl, 1 mM CaCl_2 , 10 mM glucose and 20 mM Hepes, pH 7.4) lacking calcium and containing 0.02% EDTA and were subsequently washed twice in HBSS. After stimulation by 100 nM PMA for the indicated time at room temperature, cytosolic and particulate fractions were prepared as described by Kass et al., 1989. Proteins were subjected to PKC-isoform specific antibodies according to the western blot protocol mentioned above.

Fluorescence-activated cell sorting (FACS) analysis. This analysis was based on the measurement of the DNA content of nuclei labelled with propidium iodide. For cell cycle evaluation, cells were treated as for the proliferation experiments, washed with ice-cold PBS and incubated with propidium iodide (0.05% mg/ml in 3.8 μM sodium citrate, 0.1% Triton X-100) for 15 min at room temperature in the dark. Cells then were subjected to FACS and analysis of the cell cycle was evaluated using FACSCalibur flow cytometer (Becton-

Dickinson Biosciences, San Jose, CA, USA). Data were analyzed using the Cell Quest Pro software package (BD Biosciences).

Transfection with small interference RNA (siRNA). FRO cells (100 000 cells/35 mm dish) were seeded in penicillin/streptomycin-free medium for 24 h and subsequently transfected with a negative control, or with double-stranded siRNAs (dsRNAs) purchased from Dharmacon Inc. (Dallas, TX, USA). The following targeting sequences were used: PKC α (AATCCTTGTCCAAGGAGGCTG) and PKC δ (AACCATGAGTTTATCGCCACC). dsRNAs were transfected into FRO cells using METAFECTENETM PRO (Biontex, Martinsried/Planegg, Germany) following the protocol provided by the manufacturer. 48 h after transfection, cells were stimulated with PMA in 5% lipid-stripped FBS medium. Western blot and ³H-thymidine incorporation experiments were carried out as described previously.

Microscopy. FRO cells were grown on 35-mm plates and treated with 100 nM PMA or vehicle for the indicated time, in low FBS containing medium. Light microscopic monitoring were carried out using the Axiovert 35 (Carl Zeiss, Oberkochen, Germany) microscope and analysed by the “Scion” image-processing program (Scion Corporation, MD, USA).

Statistics. Results are expressed as means $\pm$ SEM. Analysis of statistical significance was performed using Student’s *t* test for paired observations. Three or more means were tested using one-way ANOVA and Dunnett’s post hoc test. Curve fitting and statistical analyses were made using the Prism 3.03 program (GraphPad, San Diego, CA, USA). A P-value less than 0.05 was considered significant.

Results

Effects of PMA on thyroid cancer cell proliferation. In FRO thyroid cancer cells, PMA decreased the incorporation of ^3H -thymidine in a concentration- and time-dependent manner (Fig. 1A & 1B). In ARO cells, a substantially higher concentration of PMA was needed to decrease the incorporation of ^3H -thymidine (Fig. 1A). A PMA-evoked decrease in the proliferation of FRO and ML-1 cells was also seen with the celltiter assay (Fig. 1C). As some FRO cells detached after PMA treatment, we verified the results by counting all cells in a cell dish. In these experiments we observed an increase in the cell number in control dishes, whereas no increase was observed in cells incubated with 100 nM PMA for 24 h (Fig. 1 D). In all these experiments neither the phorbol ester analog 4 α -phorbol (1 μM) nor the diacylglycerol (DAG) analog 1,2-dioctanoyl-sn-glycerol (DOG, 10 μM) could inhibit FRO cell proliferation. In order to clarify the mechanism of action of the antiproliferative effect of PMA, we preincubated the cells for 1 h with the PKC inhibitors [GF 109203X (10 μM), calphostin C (100nM), rottlerin (10 μM) or GÖ 6976 (1 μM)], the MAP/ERK1/2 kinase inhibitors [PD 98059 (10 μM) or U 0126 (10 μM)], the calcium channel blocker 2APB (50 μM), the calpain inhibitors [ALLN (50 μM) or ALLM (50 μM)] or the PI-3 kinase inhibitor LY 294002 (10 μM). However, all these compounds per se decreased substantially FRO cell proliferation, and were, in addition, unable to attenuate the PMA-induced effect on cell proliferation (data not shown). Thus, the use of these inhibitors made it impossible to draw any conclusions on the mechanisms mediating the antiproliferative effect of PMA.

When cells treated with PMA for 24 h were washed and replated, the cells continued to grow with almost the same efficiency as control cells (Fig 1E). We also investigated the effects of PMA on FRO cell morphology. Treatment with PMA resulted in a rapid (within 15 min) rounding of the cells (Fig. 1F).

Effects of PMA on FRO, ARO and ML-1 cell migration. We next studied the migration of FRO, ARO and ML-1 cells and we noticed that PMA reduced the migration of cells in a different concentration profile when lipid-stripped FBS was used as a chemoattractant. The inhibitory concentrations were higher compared to those needed to attenuate the proliferation and neither the phorbol ester analog, 4 α -phorbol nor DOG could inhibit FRO cell migration toward serum (Fig. 2A & 2B).

Effects of PMA on the cell cycle and the expression levels of cell cycle regulatory proteins. To further understand by which mechanism PMA blocked cell proliferation, we next determined the cell cycle profile of FRO cells after the treatment with PMA. Interestingly, PMA induced a significant change in the distribution of the cells: an increase of the cell population in the G1 phase and a decrease in the S phase of the cell cycle (Fig. 3A & Table 1). We were unable to detect any apoptotic cells in this assay. In addition, neither 4 α -phorbol nor DOG induced significant changes in the cell cycle (Table 1). To clarify the mechanism by which PMA induced cell cycle changes, we examined the possible roles of p27^{kip1} and p21^{waf1/cip1}, two oncogenes linked to the regulation of the cell cycle (Damia and Broggini, 2004), and also cyclin D1, cyclin D2, cyclin D3, cyclin E, cdk2, cdk4, cdk6 and the Rb-protein, all of which are of importance in the regulation of the cell cycle in the G1 phase. The levels of p21^{waf1/cip1} and p27^{kip1} were significantly increased, compared with control cells. Additionally, treatment of the cells with PMA could not affect the expression levels of cyclin D1, cyclin D2, cyclin E and cdk2, but significantly decreased the levels of cyclin D3, cdk4, cdk6 and the levels of Rb-protein phosphorylation at Ser-780 and Ser-795 (Fig. 3B & Table 2).

Effects of PMA on different PKC isoforms in FRO cells. We next investigated the effects of PMA on the translocation of different PKC isoforms between the cell membrane and the

cytosol. Previously we have shown that FRO cells express four different isoforms (i.e. α , β I, δ , ζ) of PKC (Afrasiabi et al., 2006). Here we show that stimulating the cells with 100 nM PMA resulted in a rapid translocation of PKC α , PKC β I and PKC δ from the cytosolic to the particulate fractions (Fig. 4). Densitometric analysis showed that the relative distribution of PKC α to the particulate fraction was elevated by $98.0 \pm 7.1\%$ after 10 min of stimulation with PMA ($P < 0.05$), although the distribution to the cytosolic fraction was not changed. The distribution of PKC β I in the cytosolic fraction was decreased by $86.1 \pm 5.5\%$ ($P < 0.05$), whereas the distribution to the particulate fraction was increased by $79.6 \pm 12.7\%$ ($P < 0.05$) after 10 min. The distribution of PKC δ was also altered; the cytosolic fraction was reduced by $72.2 \pm 14\%$ ($P < 0.05$) and the particulate fraction increased by $91.1 \pm 4.9\%$ ($P < 0.05$), after 10 min of stimulation. PMA was without a significant effect on the distribution of the PKC ζ isoform.

The inhibitory effects of PMA in FRO cells proliferation is mediated by PKC δ . We investigated further which PKC isoform mediated the effect of PMA. Preincubation of the cells with a cell-permeant inhibitory peptide against PKC β I did not attenuate the effect of PMA on proliferation, although the distribution of PKC β I to the particulate fraction was potentially attenuated (data not shown). We next transfected the FRO cells with a negative control, or with specific dsRNAs for PKC α and PKC δ (Fig. 5 A&B), since these isoforms are known to be responsible for growth inhibitory properties in different cell types (Nakagawa et al., 2005). As it is shown in Fig. 5C, the inhibitory effects of PMA on FRO cell proliferation was reduced by more than 50% in cells transfected with PKC δ siRNA compared to PKC α -depleted and control cells, indicating that PKC δ mediates the anti-proliferative effect of PMA in FRO cells. Moreover, when cells were transfected with PKC δ siRNA, PMA was not able to

upregulate neither p27^{kip1} nor p21^{waf1/cip1}, whereas an increase was still seen in PKC α -depleted and control cells (Fig. 5D & Table 3).

Effects of PMA on the phosphorylation of MAP kinase, Akt and FOXO1. Upon short stimulation (0-60 min) of FRO cells with PMA, the phosphorylation of MAP kinase at Thr-202/Tyr-204 increased, but decreased significantly after 24 h of treatment (Fig. 6A&B). PMA was without an effect on the total amount of MAP kinase in the cells. Furthermore, the phosphorylation of Akt at Thr-308 was decreased after a 30-min stimulation (Fig. 6C). Finally, PMA stimulation resulted in an increase of the phosphorylation levels of FOXO1 at Ser-256 after a 30-min stimulation, whereas the phosphorylation was decreased after 24 h of stimulation (Fig. 6 D&E). We could not observe any PMA-evoked effects on the phosphorylation of FOXO1 at Thr-24, either at 30 min or 24 h of stimulation and were unable to detect an effect of PMA on the total amount of FOXO1 after either a 30 min- or a 24 h-stimulation (data not shown).

Discussion

In the present study we show that PMA is an inhibitor of FRO thyroid cancer cell proliferation and migration. Our results suggest that the antiproliferative effect of PMA was due to an arrest of the cells in the G1-S phase of the cell cycle. This arrest was the result of a PKC δ -mediated upregulation of the cdk inhibitory proteins p21^{waf1/cip1} and p27^{kip1} and a downregulation of cyclin D3, cdk4, and cdk6. In addition, we observed a substantial hypophosphorylation of the Rb-protein. Furthermore, our results suggest that these effects are, at least in part, mediated through an inhibition of the PI-3 kinase/Akt-FOXO and MAP kinase pathways.

Previous reports have established that PMA is a potential antiproliferative agent in different cancer cell lines, including NPA thyroid cancer cells (Frey et al., 2000; Nakagawa et

al., 2005; Koike et al., 2006; Martin et al., 2007). In these investigations, PKC α , δ and ϵ have been shown to mediate the effect of PMA. In our experiments, the translocation of PKC α , β I and δ to the cell membrane indicated a possible role for these isoforms in PMA induced signaling. Using a specific PKC β I inhibitory peptide, and siRNA against PKC α and PKC δ we showed, that in FRO cells, PKC δ is responsible for the antiproliferative effect of PMA. Although PKC δ seems to evoke a downregulation of cyclins D1 and E in several other cancer cell types (Nakagawa et al., 2005; Cerda et al., 2006; Koike et al., 2006), we could not observe any effects of PMA on cyclin D1, D2 or E. However, other investigations have stressed the importance of cyclin D3 in PI-3 kinase/Akt evoked G1-S transition (Spofford et al., 2006). Our results support the conclusion that the PI-3 kinase/Akt pathway is of importance in regulating cyclin D3. Thus, the cell signaling pathways leading to inhibition of proliferation are at least to some degree cell type specific.

Akt is a central signaling molecule in the regulation of cell proliferation and migration. Akt is constitutively activated in human cancers, including thyroid cancer, leading to the suppression of p21^{waf1/cip1} and p27^{kip1} and facilitating progression through the G1-S phase of the cell cycle (Liang and Sligerland, 2003; Shinohara et al., 2007). Thus, one mechanism by which PMA exerts its effect seems to be through inhibition of Akt, resulting in upregulation of p21^{waf1/cip1} and p27^{kip1} and arrest of the cells in the G1-S phase. Furthermore, Akt regulates the phosphorylation of the forkhead family transcription factors (FOXO) leading to their nuclear exclusion and thus inhibition of FOXO-mediated gene expression (Brunet *et al.* 1999). As the promotor of p27^{kip1} contains multiple putative FOXO binding sites and as FOXO can upregulate p27 gene expression (Medema et al., 2000), it is feasible to assume that the PMA-evoked inhibition of Akt resulted in a FOXO-mediated upregulation of p21^{waf1/cip1} and p27^{kip1}. Previous studies have also suggested that p21^{waf1/cip1} and p27^{kip1} are upregulated by PKC δ in e.g. prostate (Nagakawa et al., 2005), colon (Perletti et al., 2004), and thyroid

cancer cells (Koike et al., 2006) and that both p21^{waf1/cip1} and p27^{kip1} play an important role in attenuating thyroid cancer growth (Podtcheko et al., 2003; Yang et al., 2003).

We were able to observe a decreased phosphorylation of the Rb-protein at Ser-780 and especially Ser-795 in PMA treated cells. This is an important observation, as the Rb-protein is a central nuclear protein in regulating the progression of cells through the G1-S phase of the cell cycle (Massague, 2004). We assume that this hypophosphorylation of Rb was the result of the decrease in both cyclin D3 and cdk4/6. In SKBR-3 breast cancer cells, the PMA evoked inhibition of proliferation was mediated by a PKC δ -evoked hypophosphorylation of the Rb-protein, although the mechanism was not eluded (Yokoyama et al., 2005).

Previous studies have shown that a transient MAP kinase activation is linked to cell cycle progression, whereas sustained levels of MAP kinase activity can lead to cell growth arrest (Clark et al., 2004). Furthermore, the MAP kinase pathway is essential for G1- to S-phase progression and is associated with induction of positive regulators of the cell cycle (Meloche and Pouyssegur, 2007). Our results show that acute stimulation of the cells with PMA resulted in an increase of the phosphorylation levels of MAP kinase, while prolonged stimulation decreased the phosphorylation of this kinase. Similar results were recently also observed in FRO cells stimulated with sphingosylphosphorylcholin, a bioactive lipid that potently abrogated both migration and proliferation of FRO cells, although in these experiments the cells were arrested in the G2/M phase of the cell cycle (Afrasiabi et al., 2007). As MAP kinase is an important regulator of proliferation in several cells, including thyroid cells, this decrease probably also is of importance in the PMA-evoked inhibition of proliferation. Interestingly, although PMA evoked an acute stimulation of MAP kinase phosphorylation, we were able to observe an inhibition of ³H-thymidine incorporation already after 2 h of incubation with PMA. The results suggest that the onset of inhibition is

initiated very rapidly after stimulation. Possibly some PKC isoform other than PKC δ is mediating the early phosphorylation of MAP kinase.

We have not further investigated the mechanism by which PMA inhibited migration, as the low level of siRNA transfection precluded a more thorough investigation. However, it is tempting to speculate that PKC δ also is regulating migration in FRO cells. This isoform has been shown to be responsible for the inhibition of migration of prostate cancer cells, smooth muscle cells, and Madin Darby canine kidney cells (Kharait et al., 2006; Liu et al., 2007; Chen et al., 2007).

In conclusion, we have in the present report shown that PMA potently inhibits both the proliferation and migration of human thyroid FRO anaplastic cancer cells. At least the inhibition of proliferation seems to be mediated by PKC δ . Our results suggest that targeted activation of PKC δ could be an effective means to evoke substantial inhibition of thyroid cancer cell proliferation.

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510 Table 1. Effects of PMA, 4 α -phorbol and DOG on the cell cycle of FRO cells.

Cell cycle phase	C	PMA	4 α -phorbol	DOG
G0-G1	62.8 $\pm$ 1.6	75.3 $\pm$ 1.3**	62.9 $\pm$ 0.5	63.6 $\pm$ 0.9
S	19.1 $\pm$ 0.5	11.9 $\pm$ 0.7**	19.3 $\pm$ 0.2	19.1 $\pm$ 0.4
G ₂ -M	9.1 $\pm$ 0.6	6.2 $\pm$ 0.4**	9.3 $\pm$ 0.3	8.9 $\pm$ 0.3

511

512 Cells were treated either with vehicle (C), 100 nM PMA, 1 μ M 4 α -phorbol or 10 μ M DOG.

513 After 24 h the cells were collected and analyzed for the distribution of cells in the cell cycle

514 using FACS analysis. The data gives the mean $\pm$ SEM of three separate experiments. **, P <

515 0.01 compared with respective control value.

516 Table 2. Effect of PMA on the expression levels of the cell cycle regulatory proteins.

Protein	C	PMA
cyclin D1	1.00 ± 0.07	0.97 ± 0.06
cyclin D2	1.03 ± 0.06	1.09 ± 0.06
cyclin D3	1.00 ± 0.07	0.57 ± 0.03*
cyclin E	1.12 ± 0.17	0.94 ± 0.13
cdk2	1.03 ± 0.03	0.98 ± 0.05
cdk4	1.18 ± 0.07	0.82 ± 0.05*
cdk6	1.15 ± 0.05	0.71 ± 0.06*
p21	0.68 ± 0.15	1.32 ± 0.17*
p27	0.77 ± 0.08	1.3 ± 0.12*
pRb (Ser780)	1.16 ± 0.01	0.84 ± 0.03*
pRb (Ser795)	1.33 ± 0.18	0.66 ± 0.13*

517

518 FRO cells were treated with vehicle (C), or with 100 nM PMA for 24 h and western blot
 519 analysis were performed. Densitometric results were corrected by normalization for β -actin
 520 expression except with pRb (Ser780) and pRb (Ser795) which were normalized against the
 521 total Rb. Values given are the mean $\pm$ SEM of three independent experiments. *, $P < 0.05$
 522 compared with respective control value.

Table 3. Effect of PMA on the expression levels of p21 and p27 in cells transfected with PKC α (si α) or PKC δ (si δ) siRNA.

Protein	C	PMA	si α	si α +PMA	si δ	si δ +PMA
p21	1 $\pm$ 0.04	1.7 $\pm$ 0.1*	1.0 $\pm$ 0.06	1.74 $\pm$ 0.03**	1.03 $\pm$ 0.02	1.0 $\pm$ 0.04
p27	1 $\pm$ 0.0.25	1.78 $\pm$ 0.07*	0.93 $\pm$ 0.07	1.56 $\pm$ 0.08*	1.04 $\pm$ 0.1	1.03 $\pm$ 0.03

Control cells (C), cells transfected with PKC α (si α) or PKC δ (si δ) siRNA were treated with vehicle or with 100 nM PMA and cellular proteins were extracted after 24 h. Western blot analysis were performed using antibodies against p27^{kip1} and p21^{waf1/cip1}. Densitometric results were corrected by normalization for β -actin. Values given are the mean $\pm$ SEM of three independent experiments. *, P < 0.05, **, P < 0.01 compared to the respective control value.

Legands to the figures

Figure 1: Antiproliferative effects of PMA in FRO, ARO and ML-1 thyroid cancer cells. A. FRO and ARO cells were grown in 35-mm dishes under conditions of low (5% lipid-stripped) serum and after treatment with vehicle (0) or with different concentrations of PMA, 1 μ M 4 α -phorbol (α -ph) or 10 μ M DOG for 24 h, 3 H-thymidine incorporation assay was performed as described in the methods section. B. FRO cells were treated with 100 nM PMA and the 3 H-thymidine incorporation assay was performed after 2, 4 and 8 h. C. In the Celltiter assay, FRO and ML-1 cells were grown on 96-wells plates and treated with vehicle (0) or with different doses of PMA, 1 μ M 4 α -phorbol or 10 μ M DOG for 24 h and the absorbance at 540 nm was measured as described in the methods section. D. PMA reduces FRO cell number. Cell counting was performed after a 24 h-stimulation with either vehicle (C), 100 nM PMA, 1 μ M 4 α -phorbol (α -ph) or 10 μ M DOG. Data are expressed as percent proliferated cells compared to untreated controls. The bars give the mean $\pm$ SEM of 4-6 experiments (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$). E. FRO cells were treated with vehicle or with 100 nM of PMA, and after 24 h, cells were washed twice with PBS, counted and then replated and the 3 H-thymidine incorporation assay was performed after 24 h, 48 h and 96 h respectively, as described in the methods section. Data are expressed as percent proliferated cells compared to untreated controls. The bars give the mean $\pm$ SEM of 4-6 experiments (*, $P < 0.05$). F. Effects of PMA on FRO cell morphology. FRO cells were grown as for the proliferation experiment in 35 mm culture dishes and treated with vehicle (C) or 100 nM PMA (PMA). The cells were monitored microscopically prior to (0 min) and after 3 min, 5 min, 15 min and 30 min of stimulation, and images were recorded with a CCD camera. Each experiment was performed 3-6 times.

Figure 2: Antimigratory effect of PMA on FRO, ARO and ML-1 thyroid cancer cell migration. A. FRO cells were grown on Transwell inserts coated with collagen I as described in the methods section, and after treatment with vehicle (0), 10 nM or 100 nM PMA, 1 μ M 4 α -phorbol, or 10 μ M DOG, cells that had migrated towards lipid-stripped FBS were counted. ML-1 and ARO cells were grown on Transwell inserts coated with collagen I, and after treatment with vehicle (0), or the indicated concentration of PMA, cells that had migrated towards lipid-stripped FBS were counted. Each experiment was performed 3-6 times. Data are expressed as percent migrated cells compared to untreated controls (*, $P < 0.05$, **, $P < 0.01$). B. Representative microscopic images of the Transwell inserts of FRO cells treated with vehicle (C), or with 100 nM PMA. The arrow indicates some migrated cells after PMA treatment.

Figure 3: PMA evokes cell cycle changes. A. FRO cells were grown on 35-mm plates and treated with vehicle (C), or with 100 nM PMA for 24 h, and then incubated with propidium iodide and analysed by FACS for cell cycle measurements as described in the methods section. Axes were scaled differently to show sub-G1 population of cells. Each experiment was performed 4-6 times. B. Effects of PMA on the levels and activity of cell cycle regulatory proteins in FRO thyroid cancer cells. Cells were treated with vehicle (C) or 100 nM PMA, and cellular proteins were extracted after 24 h. A total of 15 μ g cell extract protein isolated from the treated and the untreated cells was subjected to SDS-PAGE and immunoblotted with antibodies against cyclin D1, cyclin D2, cyclin D3, cyclin E, cdk2, cdk4, cdk6, p21^{waf1/cip1}, p27^{kip1}, phospho-Rb (Ser780), phospho-Rb (Ser795) and total Rb. Filters were reblotted with an anti- β -actin antibody to normalize for protein loading. Western blot data presented are representative of 3 separate experiments.

Figure 4: Translocation of different PKC isoforms after stimulation with PMA. The cells were stimulated with 100 nM PMA and fractionated as described in the methods section. The blots show the effect of PMA on the distribution of PKC α , PKC β I, PKC δ and PKC ζ between the cytosolic and the particulate fractions before (0 min) and after 1 min, 5 min and 10 min stimulation. Each experiment was performed 3 times.

Figure 5: The inhibitory effect of PMA on proliferation is mediated by PKC δ . A. Cells were grown on 35 mm culture dishes for 24 h and subsequently transfected with a negative control dsRNAs (C-), or with PKC α and PKC δ dsRNAs (Si). After 24 h proteins were extracted from the transfected and nontransfected cells (C) and western blots were carried out using antibodies against PKC α and PKC δ . Filters were reblotted with an anti- β -actin antibody to normalize for protein loading. B. Summary of the densitometric analysis of the transfection experiments. Western blot data presented are representative of three separate experiments. (*, $P < 0.05$, **, $P < 0.01$). C. Negative control dsRNAs cells (C), or cells transfected with specific dsRNAs for either PKC α (α) and PKC δ (δ) were treated with 100 nM PMA and the ^3H -thymidine incorporation assay was performed after 24 h as described in the methods section. Data are expressed as percent proliferated cells compared to untreated controls. The bars give the mean $\pm$ SEM of 4-6 experiments (***, $P < 0.001$ compared with control cells). D. Control cells (C), cells transfected with PKC α (si α) or PKC δ (si δ) siRNA were treated with vehicle (-) or 100 nM PMA (+) and cellular proteins were extracted after 24 h. Western blott analysis were performed using antibodies against p27^{kip1} and p21^{waf1/cip1}. Anti- β -actin antibody was used to normalize for protein loading. Each experiment was performed 3 times.

Figure 6: Effects of PMA on the phosphorylation levels of MAP kinase, Akt and FOXO in FRO thyroid cancer cells. A. Cells were treated with vehicle (0), or 100 nM PMA in medium

605 containing 5% lipid-stripped FBS and cellular proteins were extracted after 5, 15, 30, and 60
606 min. B. Cells were treated with vehicle (0), or 100 nM PMA in medium containing 5% lipid-
607 stripped FBS and cellular proteins were extracted after 24 h. A total of 15 µg cell extract
608 protein isolated from the treated and the untreated cells was subjected to SDS-PAGE and
609 immunoblotted with antibodies against phospho-p44/42 MAP kinase (Thr-202/Tyr-204), and
610 p44/42 MAP kinase. C. Cells were treated with vehicle (C), or 100 nM PMA for 30 min and
611 antibodies against phospho-Akt (Thr-308) and Akt were used. D. Cells were treated with
612 vehicle (C), or 100 nM PMA for 30 min and antibodies against phospho-FOXO1 (Ser-256)
613 and FOXO1 were used as described previously. E. Cells were treated with vehicle (C), or 100
614 nM PMA for 24 h and antibodies against phospho-FOXO1 (Ser-256) and FOXO1 were used.
615 Densitometric analysis was performed and data was normalised against cells treated with
616 vehicle (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$). Western blot data presented are
617 representative of 3 separate experiments.

A

B