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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 Brogini, 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).

73 The phosphoinositide-3 kinase (PI-3 kinase)/Akt pathway is also an important regulator
74 of proliferation and has been implicated in thyroid tumorigenesis and particularly in the
75 inactivation of p21^{Waf1/Cip1} (Liang and Singerland 2003; Shinohara et al., 2007). Furthermore
76 the forkhead box class-O (FOXO) transcription factors play critical roles in a variety of
77 physiological and pathological processes including cancer and are subject to negative
78 regulation by the PI-3 kinase/Akt signal transduction pathway (Arden, 2006; Myatt and Lam,
79 2007). FOXO can also upregulate p27 and p21 gene expression (Medema et al., 2000; Dehan
80 and Pagano, 2005).

81 PKC is involved in the control of the mitogen-activated protein (MAP) kinase pathway,
82 which is responsible for controlling of diverse cellular responses, such as proliferation,
83 differentiation and cell death (Pettersson et al., 2004). In NPA thyroid cancer cells, PKC δ
84 activation has been shown to induce growth arrest by a mechanism dependent on MAP kinase
85 (Koike et al., 2006). Recently we have shown that the MAP kinase pathway is an important
86 component in the regulation of thyroid cancer cell proliferation (Afrasiabi et al., 2007) and in
87 our previous studies we were able to show some implications for the role of PKC in thyroid
88 cancer cell signaling (Afrasiabi et al., 2006). In addition to regulating proliferation, cell
89 migration is another important biological process which is regulated by PKC (Nishizuka
90 1988). While PKC δ inhibition has been shown to limit the migration of prostate cancer cells
91 (Kharait et al., 2006), PMA has been indicated as a negative regulator of breast cancer cells
92 migration by targeting PKC α (Gauthier et al., 2003).

93 Previous studies have shown that by activation of PKC in dog thyroid cells, or in rat
94 FRTL-5 thyroid cells, PMA promotes cell proliferation (Roger et al., 1986; Takada et al.,
95 1988; Lombardi et al., 1988). In human cells in culture, a biphasic effect is observed: in short-
96 term cultures, PMA inhibits proliferation, but in cells grown for longer periods, PMA

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

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

103

104 **Materials and methods**

105 *Materials.* RPMI 1640 medium (without L-glutamine) and non-essential aminoacids were
106 from Cambrex (Verviers, Belgium). L-glutamine, foetal bovine serum (FBS), penicillin and
107 streptomycin were from GIBCO (Grand Island, NY, USA). DMEM (Dulbecco's modified
108 Eagle's medium), phorbol 12-myristate 13-acetate (PMA), 4 α -phorbol, DOG and propidium
109 iodide were from Sigma (St.Louis, MO, USA). GF 109203X, calphostin C, rottlerin, PD
110 98059, UO 126, LY 294002 and GÖ 6976, ALLM and ALLN were from Calbiochem
111 (Darmstadt, Germany). Celltiter 96 solution cell proliferation assay was purchased from
112 Promega corporation (Madison, WI, USA). Human Collagen type I was from Becton
113 Dickinson Biosciences (Bedford, MA, USA). Primary antibodies against p21^{waf1/cip1}, p27^{kip1},
114 cyclin D1, cyclin D2, cyclin D3, cyclin E, cdk2, cdk4, cdk6, Rb, phospho-Rb (Ser-780),
115 phospho-Rb (Ser-795), p44/42 MAP kinase, phospho-p44/42 MAP kinase (Thr-202/Tyr-204),
116 Akt, phospho-Akt (Ser-473), phospho-Akt (Thr-308), FoxO1, phospho-FoxO1 (Ser-256),
117 phospho-FoxO1 (Thr-24) and β -actin were obtained from Cell Signaling Technology
118 (Danvers, MA, USA). Isoenzyme-specific PKC antibodies were from Santa Cruz
119 Biotechnology (Santa Cruz, CA, USA). PKC β I specific inhibitory peptide (KIBI31-1) was
120 obtained from KAI Pharmaceuticals (South San Fransisco, CA, USA). The secondary

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

123

124 *Cell culture.* The FRO and ARO human anaplastic thyroid cancer cell lines were a generous
125 gift from Dr. James Fagin (Memorial Sloan-Kettering Cancer Center, NY, USA). The ML-1
126 follicular thyroid cancer cells were provided generously by Dr. Johann Schönberger
127 (University of Rosenberg, Germany). FRO and ARO cells were cultured in RPMI 1640
128 medium and ML-1 cells were cultured in DMEM medium supplemented with 10% FBS, 1%
129 non-essential aminoacids, 2 mM L- glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin
130 and were grown in a water-saturated atmosphere containing 5% CO₂ and 95% air at 37 °C.
131 The day before an experiment the medium was removed and replaced with RPMI containing
132 5% dextran-treated-charcoal-stripped FBS.

133

134 *³H-thymidine incorporation assay.* Cell proliferation was determined using ³H-thymidine
135 incorporation (Amersham, Buckinghamshire, UK). For the assay 50 000 cells were cultured
136 for 48 h on 35-mm plates in a water-saturated atmosphere containing 5% CO₂ and 95% air at
137 37 °C followed by 24 h of treatment with the indicated concentrations of PMA. At the day of
138 the experiment, the cultures were pulsed with ³H-thymidine (0.4 µCi/ml) for 4 h of
139 incubation. Cells were washed three times with ice cold PBS followed by a 10 min
140 incubation with 5% perchloric acid and another 10 min with 0.1 N NaOH. Cells were
141 harvested and radioactivity was counted using a Wallac 1410 liquid scintillation counter
142 (Wallac OY, Turku, Finland). ³H-thymidine uptake was expressed as mean counts per minute
143 (cpm) of triplicate samples. The results were confirmed by cell counting using trypan blue.

144

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

150

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

163

164 *Western blot analysis.* FRO cells were cultured in 5% lipid stripped FBS medium for 1 day
165 and stimulated with 100 nM PMA for the indicated time. After stimulation, FRO cells were
166 washed 3 times with ice-cold PBS and lysed in cell lysis buffer [10mM Tris (pH 7.7), 150
167 mM NaCl, 7 mM EDTA, 0.5% NP-40, 0.2 mM PMSF (phenylmethylsulfonyl fluoride) and
168 0.5 µg/ml leupeptin], the lysate was centrifuged at 13 000 rpm for 15 min at +4 °C and the
169 supernatant was collected. The protein concentration was determined using the BCATM Protein

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

180

181 *Western blot of PKC isoenzymes.* Cells were harvested in HEPES-buffered salt solution
182 (HBSS; 118 mM NaCl, 4.6 mM KCl, 1 mM CaCl_2 , 10 mM glucose and 20 mM HEPES, pH
183 7.4) lacking calcium and containing 0.02% EDTA and were subsequently washed twice in
184 HBSS. After stimulation by 100 nM PMA for the indicated time at room temperature,
185 cytosolic and particulate fractions were prepared as described by Kass et al., 1989. Proteins
186 were subjected to PKC-isoform specific antibodies according to the western blot protocol
187 mentioned above.

188

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

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

197

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

208

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

213

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

219

220 **Results**

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

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

244

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

251

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

267

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

270 cytosol. Previously we have shown that FRO cells express four different isoforms (i.e. α , β I,
271 δ , ζ) of PKC (Afrasiabi et al., 2006). Here we show that stimulating the cells with 100 nM
272 PMA resulted in a rapid translocation of PKC α , PKC β I and PKC δ from the cytosolic to the
273 particulate fractions (Fig. 4). Densitometric analysis showed that the relative distribution of
274 PKC α to the particulate fraction of was elevated by $98.0\pm 7.1\%$ after 10 min of stimulation
275 with PMA ($P < 0.05$), although the distribution to the cytosolic fraction was not changed. The
276 distribution of PKC β I in the cytosolic fraction was decreased by $86.1\pm 5.5\%$ ($P < 0.05$),
277 whereas the distribution to the particulate fraction was increased by $79.6\pm 12.7\%$ ($P < 0.05$)
278 after 10 min. The distribution of PKC δ was also altered; the cytosolic fraction was reduced by
279 $72.2\pm 14\%$ ($P < 0.05$) and the particulate fraction increased by $91.1\pm 4.9\%$ ($P < 0.05$), after 10
280 min of stimulation. PMA was without a significant effect on the distribution of the PKC ζ
281 isoform.

282

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

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

296 *Effects of PMA on the phosphorylation of MAP kinase, Akt and FOXO1.* Upon short
297 stimulation (0-60 min) of FRO cells with PMA, the phosphorylation of MAP kinase at Thr-
298 202/Tyr-204 increased, but decreased significantly after 24 h of treatment (Fig. 6A&B). PMA
299 was without an effect on the total amount of MAP kinase in the cells. Furthermore, the
300 phosphorylation of Akt at Thr-308 was decreased after a 30-min stimulation (Fig. 6C).
301 Finally, PMA stimulation resulted in an increase of the phosphorylation levels of FOXO1 at
302 Ser-256 after a 30-min stimulation, whereas the phosphorylation was decreased after 24 h of
303 stimulation (Fig. 6 D&E). We could not observe any PMA-evoked effects on the
304 phosphorylation of FOXO1 at Thr-24, either at 30 min or 24 h of stimulation and were unable
305 to detect an effect of PMA on the total amount of FOXO1 after either a 30 min- or a 24 h-
306 stimulation (data not shown).

307

308 **Discussion**

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

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

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

331 Akt is a central signaling molecule in the regulation of cell proliferation and migration.
332 Akt is constitutively activated in human cancers, including thyroid cancer, leading to the
333 suppression of p21^{waf1/cip1} and p27^{kip1} and facilitating progression through the G1-S phase of
334 the cell cycle (Liang and Sligerland, 2003; Shinohara et al., 2007). Thus, one mechanism by
335 which PMA exerts its effect seems to be through inhibition of Akt, resulting in upregulation
336 of p21^{waf1/cip1} and p27^{kip1} and arrest of the cells in the G1-S phase. Furthermore, Akt regulates
337 the phosphorylation of the forkhead family transcription factors (FOXO) leading to their
338 nuclear exclusion and thus inhibition of FOXO-mediated gene expression (Brunet *et al.*
339 1999). As the promotor of p27^{kip1} contains multiple putative FOXO binding sites and as
340 FOXO can upregulate p27 gene expression (Medema et al., 2000), it is feasible to assume that
341 the PMA-evoked inhibition of Akt resulted in a FOXO-mediated upregulation of p21^{waf1/cip1}
342 and p27^{kip1}. Previous studies have also suggested that p21^{waf1/cip1} and p27^{kip1} are upregulated
343 by PKC δ in e.g. prostate (Nagakawa et al., 2005), colon (Perletti et al., 2004), and thyroid

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

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

353 Previous studies have shown that a transient MAP kinase activation is linked to cell
354 cycle progression, whereas sustained levels of MAP kinase activity can lead to cell growth
355 arrest (Clark et al., 2004). Furthermore, the MAP kinase pathway is essential for G1- to S-
356 phase progression and is associated with induction of positive regulators of the cell cycle
357 (Meloche and Pouyssegur, 2007). Our results show that acute stimulation of the cells with
358 PMA resulted in an increase of the phosphorylation levels of MAP kinase, while prolonged
359 stimulation decreased the phosphorylation of this kinase. Similar results were recently also
360 observed in FRO cells stimulated with sphingosylphosphorylcholin, a bioactive lipid that
361 potently abrogated both migration and proliferation of FRO cells, although in these
362 experiments the cells were arrested in the G2/M phase of the cell cycle (Afrasiabi et al.,
363 2007). As MAP kinase is an important regulator of proliferation in several cells, including
364 thyroid cells, this decrease probably also is of importance in the PMA-evoked inhibition of
365 proliferation. Interestingly, although PMA evoked an acute stimulation of MAP kinase
366 phosphorylation, we were able to observe an inhibition of ³H-thymidine incorporation
367 already after 2 h of incubation with PMA. The results suggest that the onset of inhibition is

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

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

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

381

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389

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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.

523 Table 3. Effect of PMA on the expression levels of p21 and p27 in cells transfected with
 524 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

525

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

531 Legands to the figures

532 *Figure 1:* Antiproliferative effects of PMA in FRO, ARO and ML-1 thyroid cancer cells. A.
533 FRO and ARO cells were grown in 35-mm dishes under conditions of low (5% lipid-stripped)
534 serum and after treatment with vehicle (0) or with different concentrations of PMA, 1 μ M 4 α -
535 phorbol (α -ph) or 10 μ M DOG for 24 h, 3 H-thymidine incorporation assay was performed as
536 described in the methods section. B. FRO cells were treated with 100 nM PMA and the 3 H-
537 thymidine incorporation assay was performed after 2, 4 and 8 h. C. In the Celltiter assay,
538 FRO and ML-1 cells were grown on 96-wells plates and treated with vehicle (0) or with
539 different doses of PMA, 1 μ M 4 α -phorbol or 10 μ M DOG for 24 h and the absorbance at 540
540 nm was measured as described in the methods section. D. PMA reduces FRO cell number.
541 Cell counting was performed after a 24 h-stimulation with either vehicle (C), 100 nM PMA, 1
542 μ M 4 α -phorbol (α -ph) or 10 μ M DOG. Data are expressed as percent proliferated cells
543 compared to untreated controls. The bars give the mean \pm SEM of 4-6 experiments (*, $P <$
544 0.05, **, $P <$ 0.01, ***, $P <$ 0.001). E. FRO cells were treated with vehicle or with 100 nM of
545 PMA, and after 24 h, cells were washed twice with PBS, counted and then replated and the
546 3 H-thymidine incorporation assay was performed after 24 h, 48 h and 96 h respectively, as
547 described in the methods section. Data are expressed as percent proliferated cells compared to
548 untreated controls. The bars give the mean \pm SEM of 4-6 experiments (*, $P <$ 0.05). F. Effects
549 of PMA on FRO cell morphology. FRO cells were grown as for the proliferation experiment
550 in 35 mm culture dishes and treated with vehicle (C) or 100 nM PMA (PMA). The cells were
551 monitored microscopically prior to (0 min) and after 3 min, 5 min, 15 min and 30 min of
552 stimulation, and images were recorded with a CCD camera. Each experiment was performed
553 3-6 times.
554

555 *Figure 2: Antimigratory effect of PMA on FRO, ARO and ML-1 thyroid cancer cell*
556 *migration. A. FRO cells were grown on Transwell inserts coated with collagen I as described*
557 *in the methods section, and after treatment with vehicle (0), 10 nM or 100 nM PMA, 1 μ M*
558 *4 α -phorbol, or 10 μ M DOG, cells that had migrated towards lipid-stripped FBS were counted.*
559 *ML-1 and ARO cells were grown on Transwell inserts coated with collagen I, and after*
560 *treatment with vehicle (0), or the indicated concentration of PMA, cells that had migrated*
561 *towards lipid-stripped FBS were counted. Each experiment was performed 3-6 times. Data are*
562 *expressed as percent migrated cells compared to untreated controls (*, P < 0.05, **, P < 0.01).*
563 *B. Representative microscopic images of the Transwell inserts of FRO cells treated with*
564 *vehicle (C), or with 100 nM PMA. The arrow indicates some migrated cells after PMA*
565 *treatment.*

566
567 *Figure 3: PMA evokes cell cycle changes. A. FRO cells were grown on 35-mm plates and*
568 *treated with vehicle (C), or with 100 nM PMA for 24 h, and then incubated with propidium*
569 *iodide and analysed by FACS for cell cycle measurements as described in the methods*
570 *section. Axes were scaled differently to show sub-G1 population of cells. Each experiment*
571 *was performed 4-6 times. B. Effects of PMA on the levels and activity of cell cycle regulatory*
572 *proteins in FRO thyroid cancer cells. Cells were treated with vehicle (C) or 100 nM PMA,*
573 *and cellular proteins were extracted after 24 h. A total of 15 μ g cell extract protein isolated*
574 *from the treated and the untreated cells was subjected to SDS-PAGE and immunoblotted with*
575 *antibodies against cyclin D1, cyclin D2, cyclin D3, cyclin E, cdk2, cdk4, cdk6, p21^{waf1/cip1},*
576 *p27^{kip1}, phospho-Rb (Ser780), phospho-Rb (Ser795) and total Rb. Filters were reblotted with*
577 *an anti- β -actin antibody to normalize for protein loading. Western blot data presented are*
578 *representative of 3 separate experiments.*

579

580 *Figure 4:* Translocation of different PKC isoforms after stimulation with PMA. The cells
581 were stimulated with 100 nM PMA and fractionated as described in the methods section. The
582 blots show the effect of PMA on the distribution of PKC α , PKC β I, PKC δ and PKC ζ
583 between the cytosolic and the particulate fractions before (0 min) and after 1 min, 5 min and
584 10 min stimulation. Each experiment was performed 3 times.

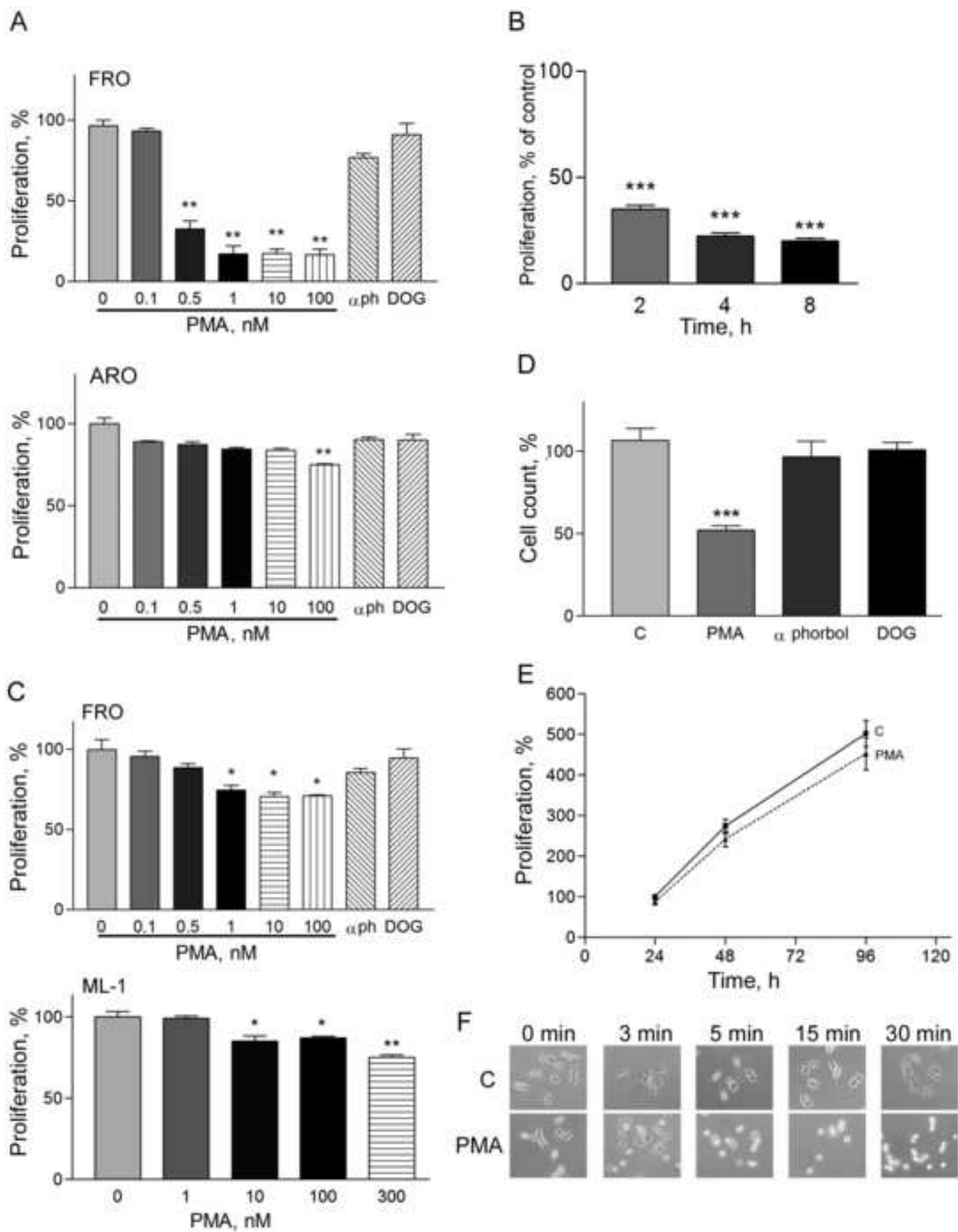
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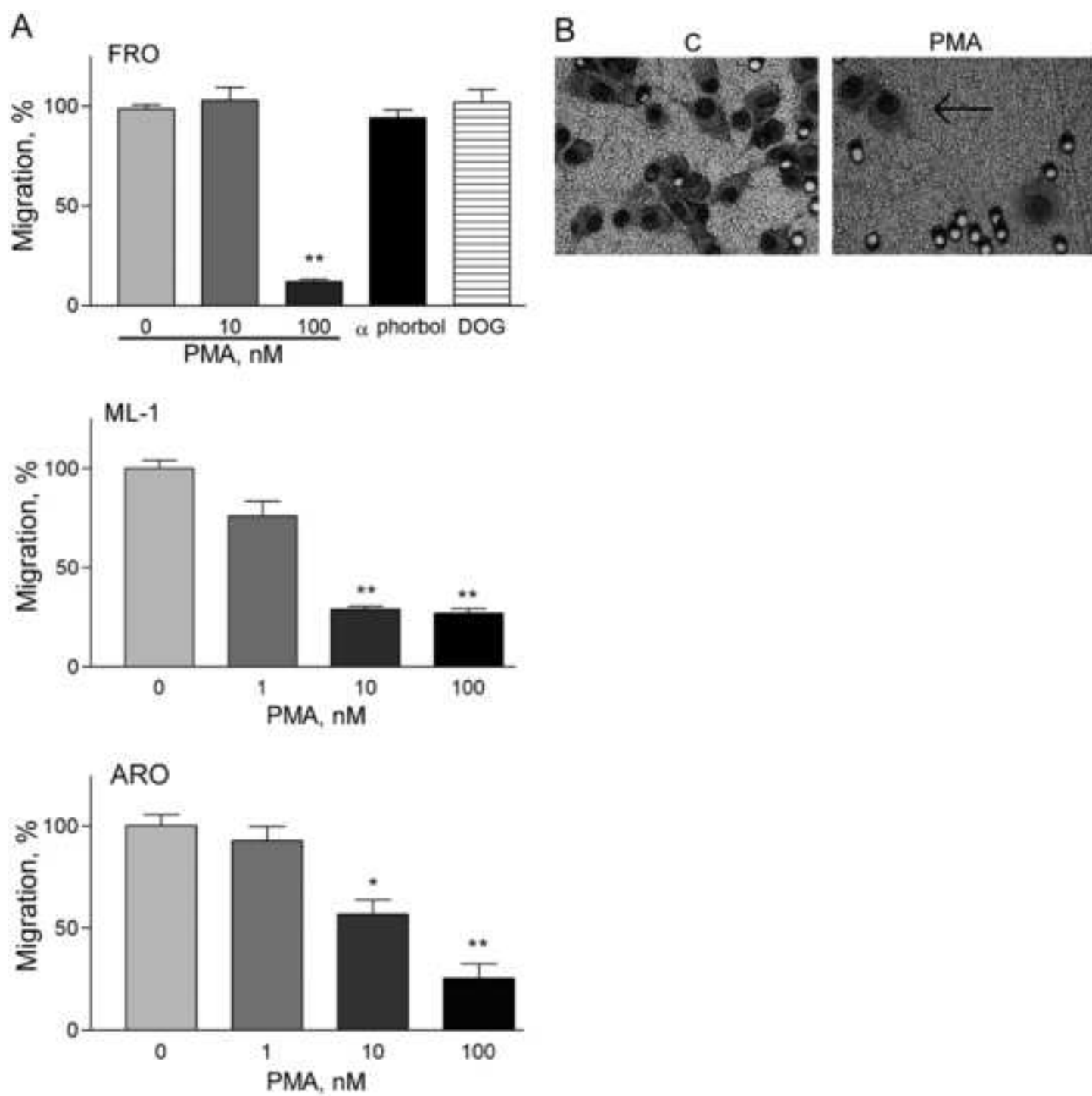
586 *Figure 5:* The inhibitory effect of PMA on proliferation is mediated by PKC δ . A. Cells were
587 grown on 35 mm culture dishes for 24 h and subsequently transfected with a negative control
588 dsRNAs (C-), or with PKC α and PKC δ dsRNAs (Si). After 24 h proteins were extracted from
589 the transfected and nontransfected cells (C) and western blots were carried out using
590 antibodies against PKC α and PKC δ . Filters were reblotted with an anti- β -actin antibody to
591 normalize for protein loading. B. Summary of the densitometric analysis of the transfection
592 experiments. Western blot data presented are representative of three separate experiments. (*,
593 $P < 0.05$, **, $P < 0.01$). C. Negative control dsRNAs cells (C), or cells transfected with
594 specific dsRNAs for either PKC α (α) and PKC δ (δ) were treated with 100 nM PMA and the
595 3 H-thymidine incorporation assay was performed after 24 h as described in the methods
596 section. Data are expressed as percent proliferated cells compared to untreated controls. The
597 bars give the mean \pm SEM of 4-6 experiments (***, $P < 0.001$ compared with control cells).
598 D. Control cells (C), cells transfected with PKC α (si α) or PKC δ (si δ) siRNA were treated
599 with vehicle (-) or 100 nM PMA (+) and cellular proteins were extracted after 24 h. Western
600 blott analysis were performed using antibodies against p27^{kip1} and p21^{waf1/cip1}. Anti- β -actin
601 antibody was used to normalize for protein loading. Each experiment was performed 3 times.

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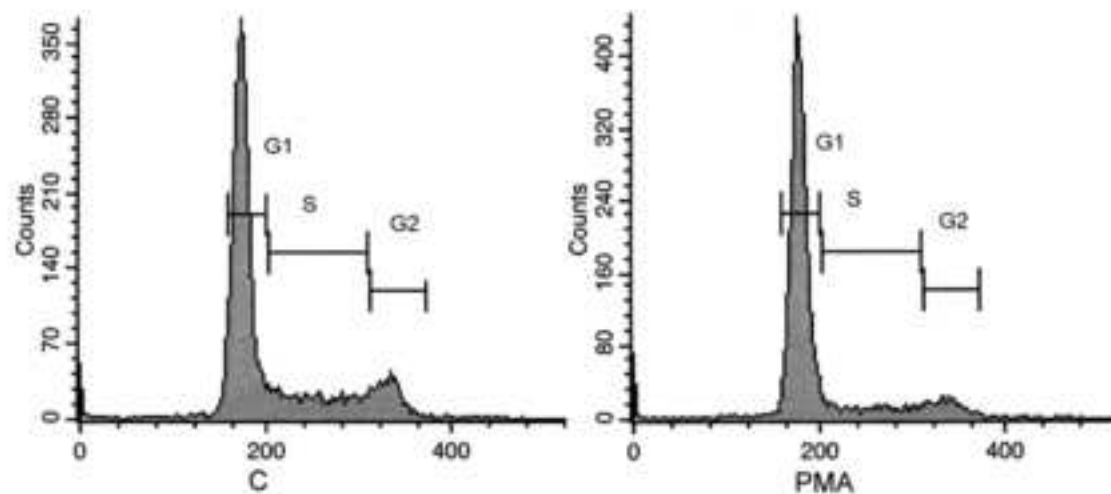
603 *Figure 6:* Effects of PMA on the phosphorylation levels of MAP kinase, Akt and FOXO in
604 FRO thyroid cancer cells. A. Cells were treated with vehicle (0), or 100 nM PMA in medium

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 μ g 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

