Phorbol 12-myristate 13-acetate inhibits epidermal growth factor signalling in human keratinocytes, leading to decreased ornithine decarboxylase activity

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Several studies have suggested that murine and human keratinocytes respond differently to phorbol 12-myristate 13-acetate (PMA). Using an *in vitro* assay, we found that in contrast to its effect on murine skin, PMA did not induce ornithine decarboxylase (ODC) activity in human skin biopsies. To explore the signalling induced by PMA and to determine whether an *in vitro* culture system could be used to predict biological activity of retinoids in human keratinocytes, we studied a simian virus 40 (SV40)-transformed human keratinocyte cell line. Epidermal growth factor (EGF) stimulates ODC activity and increases the steady-state level of ODC mRNA in a dose- and time-dependent manner in these cells [Prystowsky, Clevenger and Zheng (1993) *Exp. Dermatol.* 2, 125–132]. In this report, 10^{-10} M– 10^{-7} M PMA induced ODC mRNA and enzyme synthesis at 7 h, but did not significantly induce ODC activity and inhibited the EGF induction of ODC activity. To explore the mechanism whereby PMA interfered with EGF signalling, the effect of PMA on EGF binding to its cell-surface receptor was studied; acute

treatment with PMA (within 7 h) decreased EGF binding to 41–57% of the baseline level. In contrast, chronic treatment with PMA (24 h) increased EGF binding to 156% of the baseline level and was associated with an increase in quantity of EGF receptor protein. Protein kinase C (PKC) activation correlated with the acute decrease in EGF binding following PMA treatment. In summary, PMA induced ODC mRNA and ODC enzyme synthesis, while steady-state levels of immunoprecipitable ODC enzyme protein and ODC activity were not increased, demonstrating possible increased turnover of ODC enzyme protein. Additionally, PMA inhibited the induction of ODC by EGF through decreased EGF binding, possibly mediated by PKC activation. Finally treatment of the keratinocytes with retinoids including etretinate, Ro13-7410, etarotene, Ro40-8757, 13-*cis*-retinoic acid, and acitretin blocked the PMA induction of ODC mRNA, suggesting this *in vitro* model could be a valuable screening assay for predicting biological activity in humans.

INTRODUCTION

The tumour promoter phorbol 12-myristate 13-acetate (PMA) causes epidermal hyperproliferation and ornithine decarboxylase (ODC) induction when topically applied to murine skin [1]. The induction of ODC in murine skin by phorbol esters (PMA) and the inhibition of this phenomenon by retinoids [2,3] has formed the basis for screening retinoid compounds for biological activity [4]; retinoids are commonly used as agents for the prevention or treatment of hyperproliferative skin diseases including skin cancer [5] and psoriasis [6]. Because it was reported that PMA induced ODC *in vitro* in heat-split human skin biopsies [7], this suggested the potential to use this model to study the influence of retinoids on signalling pathways from tumour promoters in human skin cells *in vitro*. In preliminary studies, we found that while epidermal growth factor (EGF) induced ODC activity in both murine and human punch biopsies, PMA induced ODC activity in murine but not human skin biopsies. Thus, from these and other comparative studies reported by others, an important species difference is evident in phorbol ester signalling that leads to ODC induction [8–10]. The studies described here were undertaken to define in greater detail the signalling effects of phorbol esters in human skin cells. We utilized a transformed cell

line that has a constitutively activated proliferative capacity which may, in some ways, more closely simulate keratinocytes found in hyperproliferative skin disorders than normal primary cultures. Further definition of the responses of human keratinocytes to tumour-promoting agents may enhance our understanding of the role of ODC in cutaneous carcinogenesis [11] and lead to screening assays that could directly test retinoids and/or other compounds in cultured human keratinocytes instead of using live animals.

MATERIALS AND METHODS

Materials

For tissue culture, Dulbecco's modified Eagle's medium (DMEM) was obtained from GIBCO (Grand Island, NY, U.S.A.). Nu-Serum IV was obtained from Collaborative Research, Inc. (Bedford, MA, U.S.A.). Modified keratinocyte basal medium (KBM) and keratinocyte growth medium (KGM) were obtained from Clonetics (San Diego, CA, U.S.A.). The modified KBM used in all of our experiments differed from the standard formulation obtained from Clonetics in that it did not contain

Abbreviations used: ODC, ornithine decarboxylase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde phosphate dehydrogenase; KBM, keratinocyte basal medium; KGM, keratinocyte growth medium; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RAR, retinoic acid receptor.

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added putrescine or Phenol Red. We used this variant to optimize conditions for the induction of ODC because putrescine may cause feedback inhibition of ODC expression and Phenol Red is not a necessary component of the tissue culture medium. Tissue culture plasticware was obtained from Corning (Corning, NY, U.S.A.). Tissue culture-grade reagents and receptor-grade EGF were purchased from Sigma (St. Louis, MO, U.S.A.). Radio-nuclides, including L-[³⁵S]methionine, [1-¹⁴C]ornithine and carrier-free Na¹²⁵I, utilized to iodinate EGF, were obtained from Amersham (Arlington Heights, IL, U.S.A.). Iodination of EGF was performed by the chloramine-T procedure to yield a specific radioactivity of 1400–1700 Ci/mmol. A low concentration of chloramine-T (2 µg/60 µl reaction) was utilized, to optimize formation of iodinated EGF that does not cross-link to its receptors. All-*trans*-retinoic acid, referred to as retinoic acid throughout, was obtained from Sigma (St. Louis, MO, U.S.A.). All other retinoid compounds including etretinate, Ro13-7410, etarotene, Ro40-8757, 13-*cis*-retinoic acid, and acitretin, were kindly provided by Dr. Stanley Shapiro, Hoffmann-LaRoche (Nutley, NJ, U.S.A.). Murine ODC polyclonal rabbit antiserum was kindly provided by Dr. Olli Janne, University of Helsinki, Finland [12].

Cell culture

Z114, a human foreskin keratinocyte cell line immortalized with simian virus 40 (SV40), was utilized in all experiments, the details of which have been described previously [13,14]. The cells were kindly provided to us by Dr. Vittorio Defendi, New York University [15]. Stock cultures of the cells were maintained in medium consisting of DMEM supplemented with 10% (v/v) Nu-Serum IV, 0.5 µg/ml hydrocortisone, 10 ng/ml cholera toxin and 10 ng/ml EGF. Unless indicated otherwise, cells were seeded in KGM. When the cells reached 80% confluence, they were placed into modified KBM. We used KGM and KBM to eliminate the presence of serum because serum is less well defined and could contribute to variability in the PMA-induced response in the cells. When indicated, EGF and/or other agents were added to the cultures in KBM. Cultures were grown in a humidified atmosphere of 95% air/5% CO₂ at 37 °C.

EGF binding

The details of ¹²⁵I-EGF binding experiments have been described previously [16,17]. Briefly, 5 × 10⁵ c.p.m. ¹²⁵I-EGF was added in KBM (1 ml) to each 35-mm-diam. culture dish. After incubation for 3 h at 4 °C the dishes were washed three times with 2 ml of ice-cold PBS containing 0.1% (w/v) BSA. Cells were solubilized in 0.1 M NaOH and aliquots were counted in a Beckman 5500 gamma counter. Non-specific binding represented 4–8% of the total binding and was determined in parallel cultures which had a 200-fold excess of unlabelled EGF. Non-specific binding was subtracted from all points. Cell counts were determined with a haemocytometer.

ODC assay

ODC activity was determined as described previously [14]. Briefly, the release of ¹⁴CO₂ from DL-[1-¹⁴C]ornithine hydrochloride was measured. The enzymically released ¹⁴CO₂ was captured by filter paper saturated with 1 M methylbenzathonium hydroxide in methanol. Enzyme activity is expressed as pmol of CO₂ released/mg of protein per h. Soluble protein extracted from the cultures was assayed for ODC activity.

Protein kinase C (PKC) assay

To assay PKC, the 'PKC enzyme assay system' kit available from Amersham (Arlington Heights, IL, U.S.A.) was employed with slight modification as described below. Cells were rinsed and harvested in PBS at 4 °C. After homogenization of the cells in buffer containing 20 mM Tris/HCl, 5 mM EGTA, 2 mM EDTA, 0.25 mM PMSF, 5 mM 2-mercaptoethanol and 20 µg/ml leupeptin, soluble and particulate fractions were prepared. First, the homogenate was centrifuged at 100 000 g for 1 h. The supernatant was removed and represented 'cytosolic protein'. The remaining pellet was suspended in homogenization buffer with the addition of 1% Triton X-100, stirred vigorously, and centrifuged at 100 000 g for 1 h. The resulting supernatant contained 'membrane protein'. PKC was then partially purified from each fraction by DEAE-Sephacel column chromatography; semi-purified PKC was eluted with 0.3 M NaCl as described by Matsui et al. [18]. The semi-purified PKC preparations were concentrated by ultrafiltration using Amicon Centricon 30 Micro-concentrators (Amicon, Beverly, MA, U.S.A.). PKC was assayed in each fraction following the procedure outlined in the kit (see above). This assay measures the phosphotransferase activity of PKC by the enzymic transfer of the gamma phosphate group of adenosine 5'-triphosphate to a peptide which is a specific substrate for PKC [19]. PKC activity is expressed as pmol of phosphate transferred/mg of protein per min.

Protein assay

Protein content of the cell culture extracts was determined using the Bradford method [20].

Northern blot analysis

Northern blot analysis was performed for ODC and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA as described previously [13,14]. The cells were treated with PMA, EGF, retinoid compounds or vehicle as indicated in the individual experiments. Total cellular RNA was isolated by the guanidine thiocyanate/caesium chloride method and quantified by absorbance at 260 nm [21]. ODC cDNA was a generous gift from Dr. Olli Jänne [22]. GAPDH cDNA was kindly provided by Dr. M. B. Prystowsky, Albert Einstein-Montefiore Medical Center, Bronx, New York, NY, U.S.A. [23]. ODC and GAPDH mRNA were analysed by agarose/formaldehyde gel electrophoresis followed by Northern blotting. Briefly, for each lane, 20 µg of total RNA was electrophoresed in an agarose/formaldehyde gel and blotted on to a nylon membrane. The membranes were hybridized with human ODC cDNA and murine GAPDH cDNA probes (specific radioactivity 6 × 10⁸ c.p.m./µg of DNA) and exposed to X-ray film at -70 °C with an intensifying screen. The relative quantities of ODC and GAPDH mRNA were determined by laser densitometry.

EGF receptor (EGFR) immunoblotting

EGFR immunoblotting was performed as described previously [16,24]. Solubilized extracts for immunoblotting were prepared from cell cultures treated with PMA or vehicle as indicated. The samples containing 40 µg of cell extracts were electrophoresed, transferred and incubated with anti-EGFR antibody for 60 min, followed by horseradish peroxidase-conjugated secondary antibody. EGFR was immunodetected using enhanced chemiluminescence reagents, and visualized by film exposure.

Metabolic labelling and ODC immunoprecipitation

ODC immunoprecipitation was a modification of the procedure described by Ruhl et al. [10]. Cells were grown in DMEM complete medium for 2 days, then in basal medium (DMEM without added growth factors and serum) for 17 h. To assay for new synthesis of ODC protein, cells were incubated in methionine-free DMEM containing 50 ng/ml EGF, 10^{-7} M PMA or vehicle for 7 h, then 15 min before harvesting 0.1 mCi of L-[35 S]methionine was added. Cells were suspended in 500 μ l of 150 mM NaCl, 5 mM EDTA, 50 mM Tris/HCl, 0.5 % Nonidet P-40, 0.1 % SDS, pH 7.4 (buffer A), and lysed by three freeze/thaw cycles. An equal number of counts were used for immunoprecipitation by incubation of 3 ml of anti-ODC serum or normal rabbit serum for 18 h at 4 °C, followed by incubation with protein G for 1 h at room temperature. Absorbed complexes were pelleted at 14000 g , for 20 min at 25 °C, and washed three times with buffer A. Samples were boiled in loading buffer for 3 min and analysed on an SDS/10 % polyacrylamide gel. Labelled proteins were visualized by autoradiography and quantified by laser densitometry.

RESULTS

PMA inhibits the EGF induction of ODC

The effect of PMA on ODC activity was evaluated in cultured Z114 cells with and without 50 ng/ml EGF stimulation (Figure 1). When PMA (10^{-7} M to 10^{-10} M) was added and the cultures were harvested after 7 h, PMA significantly blocked the EGF induction of ODC, while EGF alone induced an 8-fold increase in ODC activity. To explore a possible direct induction of ODC by PMA, cells were exposed to 10^{-7} M PMA from 2 to 24 h in the absence of EGF stimulation; there was no significant increase in

Figure 1 Short-term treatment with PMA inhibits the EGF induction of ODC in a dose-dependent manner

Z114 cells were grown to 80% confluence in KGM. Following incubation of the cells in KBM for 24 h, they were treated with PMA (10^{-10} – 10^{-7} M) or vehicle, ethanol (1:5000) as a control, in the presence (●) or absence (○) of 50 ng/ml EGF for 7 h. ODC activity was assayed as described in the Materials and methods section. PMA significantly prevented the EGF-induced ODC activity at concentrations of PMA greater than or equal to 10^{-9} M.

Table 1 PMA does not induce ODC activity in SV40 transformed keratinocytes

Cells were grown in complete medium and placed in basal medium for 24 h, then pretreated with 10^{-7} M PMA for the indicated time and EGF (50 ng/ml) or vehicle control (1:3000 ethanol) for 7 h. ODC activity was assayed as described in the Materials and methods section. Abbreviation: N/A, not applicable.

Sample (<i>n</i> = 3)	Treatment	ODC activity (pmol of CO ₂ /mg of protein per h) (mean ± S.D.)	t-test compared with sample A)
A	Control (7 h)	338.9 ± 54.6	N/A
B	PMA (2 h)	456.1 ± 158.6	<i>P</i> > 0.10
C	PMA (7 h)	493.3 ± 188	<i>P</i> > 0.10
D	PMA (15 h)	379.1 ± 104	<i>P</i> > 0.50
E	PMA (24 h)	351.3 ± 95	<i>P</i> > 0.50
F	EGF (7 h)	3795 ± 201	<i>P</i> < 0.001

Figure 2 PMA increases the steady-state level of ODC mRNA

Z114 cells were grown in KGM until 80% confluent. They were placed into KBM for 24 h prior to treatment with 10^{-7} M PMA or vehicle (ethanol). Total RNA was prepared from cells pretreated with 10^{-7} M PMA or ethanol as follows: lane A, ethanol (1:5000) for 24 h; lane B, PMA for 2 h; lane C, PMA for 4 h; lane D, PMA for 7 h; lane E, PMA for 15 h; lane F, PMA for 24 h. Aliquots of RNA (20 μ g) were evaluated by Northern blot technique. PMA induced a maximum increase (4.4-fold) in the steady-state level of ODC mRNA by 7 h and a 2.4-fold increase of ODC mRNA was observed by 15 h after treatment with PMA. Identical data were observed in three separate experiments.

ODC activity (Table 1). The effect of PMA on ODC activity was also tested in cell-free extracts; there was no direct inhibitory effect of PMA on ODC (results not shown), suggesting that cellular signalling is involved in PMA's effect on ODC in this cell culture system.

In cells that were not co-stimulated with EGF, PMA increased ODC mRNA steady-state levels (Figure 2) in a time-dependent manner. Z114 cells were treated with 10^{-7} M PMA for up to 24 h. Northern blot analysis revealed that when compared with vehicle-treated samples, ODC mRNA steady-state levels increased 4.4-fold 7 h after treatment of the cells with PMA. By 15 h, the increase in ODC mRNA diminished to 2.4-fold.

When cultures were stimulated with EGF and/or PMA, a strong increase in steady-state ODC mRNA levels was seen when EGF, PMA, or EGF plus PMA were added to cultures (Figure 3). Because no additive effect of EGF on the PMA induction of ODC mRNA was observed, this suggests that pretreatment of the cells with PMA blocked completely the EGF induction of ODC mRNA. Thus, PMA induces ODC gene transcription but not ODC enzyme activity.

After 7 h of PMA and/or EGF treatment of cells, PMA

Figure 3 PMA inhibits the EGF induction of ODC mRNA

Z114 cells were grown in KGM to 80% confluence and placed into KBM for 24 h. They were treated with PMA for 7 h or vehicle (ethanol) in the presence or absence of 50 ng/ml EGF for 4 h as follows: lane A, vehicle (ethanol, 1:5000 dilution); lane B, 50 ng/ml EGF; lane C, 10^{-7} M PMA; lane D, 10^{-7} M PMA + 50 ng/ml of EGF. The amounts of ODC and GAPDH mRNA were determined by hybridizing the blots simultaneously with 32 P-labelled cDNA probes for each mRNA species. Laser densitometric analysis showed that 10^{-7} M PMA and 10^{-7} M PMA plus EGF treatment resulted in a 5-fold and 4.5-fold increase in ODC mRNA respectively, while EGF alone induced a 2.4-fold increase in ODC mRNA.

inhibited the EGF induction of ODC activity (Figure 1), but when Z114 cells were incubated with 10^{-7} M PMA for 24 h (prolonged treatment) there was no inhibition of the EGF induction of ODC (Figure 4), demonstrating that the inhibitory effect of PMA had dissipated at a time point coincident with PKC down-regulation (see below).

PMA treatment does not affect the steady-state immunoprecipitable ODC protein (results not shown). However, PMA caused an increase in newly synthesized, immunoprecipitable

Figure 4 Effect of prolonged treatment with PMA on ODC activity in the presence of EGF

Monolayers of Z114 cells were incubated in KBM for 24 h, then treated with 10^{-7} M PMA (○) or ethanol (1:5000) (●) for the indicated times, simultaneously with 50 ng/ml EGF. ODC activity was assayed as described in the Materials and methods section. Although 10^{-7} M PMA blocked the induction of ODC activity when incubated with EGF for 7 h, prolonged incubation (24 h) with PMA and EGF led to full EGF induction of ODC activity.

Figure 5 PMA increases ODC synthesis but not accumulation

To assay cells for new ODC new synthesis, cells were incubated in methionine-free DMEM for 7 h and treated as following: lanes A and B, 1:5000 ethanol; lane C, 1:5000 ethanol plus 50 ng/ml EGF; lane D, 10^{-7} M PMA; lane E, 50 ng/ml EGF plus 10^{-7} M PMA. Fifteen min before harvesting the cells 0.1 mCi of L-[35 S]methionine was added. Cells were lysed as described in the Materials and methods section. Lysate samples for immunoprecipitation contained an equal quantity of radioactivity. Immunoprecipitation of lysates was accomplished by incubation with either 3 ml of ODC antiserum (lanes B–E) or normal rabbit serum (lane A). Abbreviation: Kd, kDa. Immunoprecipitated ODC (51.1 kDa) is shown in lanes B, C, D and E. The intensity of ODC bands was compared with vehicle-treated cultures and quantified by densitometer. EGF and EGF plus PMA induced a 1.3-fold increase in ODC protein synthesis, while PMA alone induced a 1-fold increase compared with control (lane B).

ODC protein (Figure 5). PMA induced an increase of ODC protein similar to that observed with EGF stimulation. Combined treatment with both PMA and EGF, however, was not additive, suggesting that PMA blocks the EGF induction of ODC synthesis.

PMA alters EGFR signalling and activates PKC

To determine whether PMA interferes with the EGF induction of ODC through interference with EGF cell surface binding, the effect of PMA treatment on EGF binding to Z114 cells was evaluated. Acute treatment (within 7 h) with PMA decreased EGF binding to 41–57% of control culture values. In contrast, prolonged treatment (24 h) of cell cultures with PMA increased binding to 156% of the baseline level. Both the decrease in 125 I-EGF binding to Z114 cells that occurred when cells were incubated with PMA for 4 h and the increase in binding observed when cells were incubated with PMA for 24 h were dose-dependent (Figure 6).

The time course for the PMA induction of PKC activity was evaluated (Figures 7 and 8) to see whether PKC activation correlated with changes in EGF binding. Treatment of Z114 cells with PMA resulted in a 5-fold increase in PKC activity at 30 min and a return to basal levels by 24 h. The maximum (8-fold) induction of membranous PKC activity was obtained with 10^{-6} M PMA at 30 min. These data are consistent with the concept that PKC activation by PMA may lead to decreased EGF binding.

To determine whether the decrease or increase in EGF binding was due to an alteration in EGFR protein levels, immunoblotting was done on cell extracts from cultures treated with PMA for either 2 h or 24 h. Although no change in EGFR protein was noted at 2 h, a 2.7-fold increase in EGFR protein was detected at 24 h (Figure 9). Thus, the early decrease in EGF binding is due to inactivation of the receptor, whereas after 24 h there is an

Figure 6 Concentration-dependent effect of PMA on EGF binding within 4 h (A) and at 24 h (B)

(A) Monolayers of Z114 cells were incubated in KBM and treated with 10^{-9} M– 10^{-7} M PMA or vehicle (ethanol, 1:5000) for 4 h. ¹²⁵I-EGF binding after 3 h incubation at 4 °C was measured as described in the Materials and methods. A significant ($P < 0.05$) decrease in EGF binding was detected at concentrations equal to or greater than 10^{-8} M. (B) Monolayers of Z114 cells were incubated in KBM and treated with 10^{-10} M– 10^{-6} M PMA or vehicle (ethanol, 1:5000) for 24 h. ¹²⁵I-EGF binding after 3 h incubation at 4 °C was measured as described in the Materials and methods section. A significant ($P < 0.05$) increase in EGF binding was detected at concentrations equal to or greater than 10^{-10} M.

increase in receptor quantity, possibly due to increased production or decreased turnover.

Active retinoids inhibit PMA-induced ODC mRNA in transformed human keratinocytes

When Z114 cells were incubated with etretinate, Ro13-7410, etarotene, Ro40-8757, 13-*cis*-retinoic acid, acitretin, or all-*trans*-retinoic acid for 24 h, with the addition of PMA for the final 7 h

Figure 7 PMA induces membranous PKC in a dose-dependent manner

Z114 cells were incubated in KBM for 24 h, then treated with PMA (10^{-10} M– 10^{-6} M), or vehicle (ethanol, 1:5000) for 30 min. Cells were rinsed and harvested in PBS at 4 °C. Cytosolic and membranous enzyme extracts were prepared and PKC activity was assayed as described in the Materials and methods section. The maximum induction (8-fold) of PKC activity was obtained at 10^{-6} M PMA.

Figure 8 PMA induces PKC activity in a time-dependent manner

Z114 cells were incubated in KBM for 24 h, followed by treatment with vehicle (ethanol, 1:5000) or 10^{-7} M PMA for the indicated time. Membranous and cytosolic enzyme extracts were prepared and assayed for PKC activity as described in the Materials and methods section. 10^{-7} M PMA acutely induced cell membrane PKC activity. A maximum induction (5-fold) of membranous PKC activity was obtained at 30 min. However, the PKC activity in membrane was reduced to the basal level by 24 h.

of incubation, complete inhibition of the PMA induction of ODC mRNA was observed (Figure 10). These biologically active retinoids therefore inhibited the signalling effects of PMA in

Figure 9 Treatment of Z114 cells with PMA increases the level of EGFR at 24 h

Z114 cells were pretreated with ethanol (1:5000) for 24 h (lane B), 10^{-7} M PMA for 2 h (lane C), and 10^{-7} M PMA for 24 h (lanes A and D). Aliquots of cell extracts (40 μ g of protein) from each sample were electrophoresed, transferred and immunoblotted. Immunoblotting with EGFR antibody (lanes B, C and D) demonstrated that acute PMA treatment did not affect the quantity of EGFR protein in the cells; however, prolonged incubation with PMA (24 h) increased the amount of EGFR protein (M_r 170 kDa) by 2.7-fold. Negative control was shown in lane A, where the blot was incubated with normal goat serum (1:1000) instead of EGFR antibody.

Figure 10 Pretreatment of Z114 cells with active retinoids blocks the PMA induction of ODC mRNA

Cells were grown in KGM until 80% confluent and placed into KBM for 24 h, and treated with 10^{-6} M etretinate, Ro13-7410, etarotene, Ro40-8757, 13-*cis*-retinoic acid, acitretin, and all-*trans*-retinoic acid for 24 h with the addition of 10^{-7} M PMA for the final 7 h of incubation. A complete inhibition of the PMA induction of ODC mRNA was observed in all cases. Lanes correspond to the following treatments: lane A, vehicle control (ethanol, 1:5000 dilution); lane B, 10^{-7} M PMA; lane C, etretinate; lane D, Ro13-7410; lane E, etarotene; lane F, Ro40-8757; lane G, 13-*cis*-retinoic acid; lane H, acitretin; and lane I, all-*trans*-retinoic acid for 24 h in the presence of 10^{-7} M PMA for 7 h. Laser densitometric analysis showed that all retinoids tested completely blocked the PMA induction of ODC mRNA; however, PMA induced a 4.7-fold increase in ODC mRNA.

human keratinocytes. This suggests that this *in vitro* culture system could be utilized as a screening assay for the biological activity of retinoids.

DISCUSSION

PMA and ODC

Although it has previously been reported that PMA induces ODC in human skin biopsy specimens [7,25], recent reports suggest that ODC activity is down-regulated by PMA and UV B

radiation in human keratinocytes [9,10,14] in contrast to rodent-derived cells where both PMA and UV radiation induce ODC activity [26,27]. Furthermore, in our preliminary studies (results not presented) we were unable to demonstrate increased ODC activity following incubation of human skin with PMA. Our assay utilized whole skin biopsies (including epidermis and dermis) which may have influenced our findings compared with measurement in heat-separated epidermis in the studies of others [7]. Nevertheless, the collective evidence suggests that the signalling effects of phorbol esters in normal, immortalized, or transformed human keratinocytes are likely to be significantly different from that observed in rodent-derived keratinocytes.

In the SV40-transformed human keratinocyte Z114 cells, PMA induced ODC mRNA, but not ODC enzyme activity, although an increase in ODC enzyme synthesis was evident. It is recognized that the timing of these experiments is important; the 7 h time point was chosen to measure ODC synthesis in the presence of PMA because the other studies reported here showed that at 7 h (1) PMA treatment blocks the EGF induction of ODC activity, (2) EGF alone induces ODC activity 8-fold, (3) PMA treatment alone causes no induction of ODC activity, (4) PMA treatment alone maximally induces ODC mRNA 4.4-fold, (5) EGF binding is decreased by PMA treatment by approximately 50%, and (6) PMA causes no change in the quantity of ODC protein. Since the ODC enzyme activity was not increased by PMA (Table 1), the amount of ODC protein stayed the same, the synthesis of ODC protein was increased, and the amount of ODC mRNA was increased; these results suggest that decreased protein stability may be involved in the ODC response to PMA in human keratinocytes. Evidence for antizymes that bind to the ODC enzyme and target it for degradation and thereby reduce enzyme stability has been reported [28]. Whether this or other mechanisms are involved will require further investigation.

In contrast to our results, Fisher and colleagues noted no increase in either ODC mRNA or ODC enzyme activity in normal human epidermal keratinocytes, whereas both were increased in murine epidermal keratinocyte cultures [9]. Hickok and colleagues reported that PMA caused a marked decrease in ODC enzyme activity with no detectable effect on ODC mRNA levels in cultured normal and immortalized, but not tumorigenic, human keratinocytes. In their studies, the amount of immunoreactive protein was decreased as well as new synthesis of ODC, although no change in half-life of ODC protein was noted. Prolonged treatment of the keratinocytes with phorbol esters abolished the suppression of ODC activity by PMA [10]. In the SV40-transformed cells utilized in the studies reported here PMA induced ODC mRNA in quiescent cells (growth factor-deprived) and inhibited the induction of ODC activity in EGF-stimulated cells. It is likely that the presence of EGF or other mitogens in the growth medium utilized in the human keratinocyte studies alluded to above [9,10] was at least partially responsible for the observation of decreased ODC activity in cultures treated with PMA in growth medium compared with cultures treated with growth medium alone. Additionally, the transformed state of the Z114 cells studied here may have altered the signalling response to PMA; a study of PMA responsiveness in parental 10T1/2 fibroblasts and H-*ras*-transformed cells revealed that PMA induced ODC mRNA only in the transformed cells and not in the parental cell line [29]. Thus, the transformation process of human cells may lead to notable differences in the signalling responses to tumour promoters.

The observed lack of increased ODC activity in normal [3,9] or transformed human keratinocytes is consistent with the reported effect that PMA treatment has on inducing human keratinocyte differentiation [30]. PMA has been shown to induce differen-

tiation in the same SV40-transformed keratinocytes studied here in parallel to an inhibition in growth [31] and in human papillomavirus (HPV) type 16 immortalized human keratinocytes [32].

The decrease in EGF binding and signalling that occurs following PMA treatment of keratinocytes reported here (see below) may be importantly related to stimulating the cells to leave the cell cycle and differentiate. This response could represent a protective mechanism that would retard clonal expansion of initiated cells.

PMA and EGF binding and signalling

EGF stimulates ODC activity and increases the steady-state level of ODC mRNA in a dose- and time-dependent manner in SV40-transformed human keratinocytes (Z114) [13]. In the studies reported here, PMA inhibited the EGF induction of ODC mRNA and enzyme activity. Previous reports have not specifically addressed the effect of PMA on the EGF induction of ODC. This interruption in ODC induction is likely to be the result of the PMA-induced reduction in EGF binding. A decrease in EGF binding following treatment of cells with phorbol esters has been reported by others and was reviewed recently [33] but has not been previously linked to the significant impact this has on ODC induction presented here. The importance of ODC expression was recently illustrated by the increased frequency of skin tumours observed in transgenic mice which overexpressed ODC [34]. An enhanced expression of EGFR has been noted in hyperplastic diseases of the skin [35]. Several retinoids that decrease EGFR binding and/or the EGF induction of ODC *in vitro* in SV40-transformed keratinocytes have been found to be successful agents for the treatment of skin diseases *in vivo* [24]. Thus, analysis of EGF signalling responses in these cells may be useful to dissect signalling pathways relevant to treatment of hyperproliferative skin diseases such as psoriasis or skin cancer.

PMA and PKC

It has long been recognized that PKC is a PMA receptor and therefore important in the transcriptional regulation of PMA-inducible genes [36]. However, the mechanism whereby PKC activation leads to ODC induction has not been fully elucidated. Recent studies suggest that PKC α (calcium-dependent) and PKC δ (calcium-independent) may mediate transcription of PMA-inducible genes through both transcription-factor-AP-1 and non-AP-1 sequences [36]. ODC has a non-AP-1 PMA response element [37] and although AP-1 sites have been identified in introns 3, 5 and 11, they do not appear to be responsive to PMA when intact [38]. Acute PMA treatment stimulated PKC activation which may be responsible for directly stimulating ODC gene expression while at the same time be responsible for inactivation of the EGFR and cause decreased EGF binding. PKC is known to influence phosphorylation of the EGFR and is specifically thought to mediate phosphorylation of threonine residues and enhance inactivation of the EGFR [39], which could thereby inhibit EGF induction of ODC. After prolonged incubation of cells with PMA, PKC activity returned toward pre-treatment levels and an increase in EGF binding was observed, which coincided with the observation that PMA no longer inhibited the EGF induction of ODC. We noted a maximum increase in membranous PKC activity at 30 min with a decline to basal levels by 24 h; Matsui and colleagues noted a later rise in PKC activity (10 h) with a decline at 72 h following stimulation of cultured normal human epidermal keratinocytes with PMA [40]. Fisher et al. [9] found that treatment of adult normal human epidermal keratinocytes with PMA activated and down-regulated

PKC in both murine and human epidermal keratinocytes. These data suggest species similarity with this aspect of PMA signalling even though differences in the PKC isoenzymes in murine versus human keratinocytes were found. Thus, differences in PMA signalling between species and between immortalized versus normal human keratinocytes occur distal to PKC activation in the signalling pathway leading to ODC induction.

Retinoid screening and PMA induction of ODC mRNA

Because the hyperproliferative lesions of psoriasis and skin cancers (i.e. basal cell carcinomas and squamous cell carcinomas) are characterized by elevated ODC, the ability of retinoids to inhibit ODC induction has been taken to be an important therapeutic indicator important in drug development for these and related disorders. Thus, a frequently used screening assay for retinoids in drug development over the past two decades has been to determine whether they block the PMA induction of ODC activity in murine skin. The characteristics of the SV40-transformed human keratinocytes in their response to PMA demonstrates that ODC mRNA is reliably induced by PMA and that therapeutic retinoids block it. Z114 cells were effective for screening retinoids for biological activity by measurement of the inhibition of ODC mRNA accumulation following PMA treatment. This suggests that this assay may have predictive value for testing newer retinoids or other agents under development for the treatment of hyperproliferative skin diseases or as chemopreventive agents. It is less costly and potentially more relevant for human skin processes to measure the activity in the human cell culture system described here.

The mechanism of retinoid-induced chemoprevention and treatment of hyperproliferative skin diseases such as psoriasis has not been fully elucidated. We have reported that retinoic acid treatment leads to decreased EGF binding associated with decreased EGFR quantity in Z114 cells, and that retinoids partially inhibit the EGF induction of ODC [24]. In addition, retinoids decrease ODC expression in quiescent (not EGF-stimulated) cells [24,41]. Whether these events or others are responsible for retinoid interference with PMA induction of ODC mRNA requires further study. Recent studies suggest an interaction between PMA treatment of cells and the expression of nuclear receptors for retinoids. It has been demonstrated that PMA treatment of murine skin leads to attenuation of retinoic acid receptor (RAR) α , γ and retinoid X receptor α (RAR β was not detected in mouse epidermis) expression [42]. The specific binding of retinoic acid to RAR was decreased when PMA induced expression of ODC; it has been suggested that down-regulation of RAR(s) may be an essential component of the mechanism of mouse skin tumour promotion [42]. Treatment of fibroblasts from human skin with retinoids has been shown to up-regulate RARs [43,44], which may lead to inhibition of tumour promoter effects in the skin and chemoprevention.

Conclusions

PMA responses in SV40-transformed human keratinocytes respond similarly to normal human epidermal keratinocytes in that ODC activity is not induced and therefore support the significant difference between human and rodent species in the response to this tumour promoter. Evidence is presented supporting the concept that PKC activation in human keratinocytes (whether transformed or normal) occurs in a similar manner to that observed in rodent-derived keratinocytes, suggesting that the differences between species are distal to this signalling step. EGFR binding and signalling are impaired coincident with PKC activation by PMA, suggesting that PKC activation may in-

activate EGFR. PMA induced ODC mRNA in quiescent (no EGF added) transformed human keratinocytes, in contrast to what has been reported to occur in normal or immortalized keratinocytes treated with PMA in growth factor-containing medium, suggesting that either (1) the presence of growth medium affected the results reported for normal or immortalized keratinocytes, or (2) SV40 transformation alters phorbol ester signalling. Finally, retinoids that have been determined to exhibit biological activity in other systems were found to inhibit the PMA induction of ODC, suggesting potential utility of this culture system to test future retinoids and other agents [45] for potential in chemoprevention or treatment of hyperproliferative cutaneous disorders.

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