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Regulation of Glucocorticoid Receptor (GR) mRNA and Protein Levels by Phorbol Ester in MCF-7 Cells. Mechanism of GR mRNA Induction and Decay

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Treatment of MCF-7 cells with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (10^{-7} M) was associated with a time-dependent increase in specific binding of [^{3}H]dexamethasone (34.8 ± 4.6 fmol/mg protein after 9 h of TPA treatment compared with 16.0 ± 2.3 fmol/mg protein in control cells) as well as a transient induction in the level of glucocorticoid receptor (GR) mRNA (4- to 8-fold stimulation after 2–3 h, followed by a decline towards the control value after 6 h). In the presence of the transcription inhibitor actinomycin D (AMD) (5.0 μg/ml) the TPA-dependent induction of GR mRNA was completely abolished, and GR mRNA showed a gradual decline with a half-life of 2–3 h. In contrast, treatment with TPA and the protein synthesis inhibitor cycloheximide (50 μM) resulted in a superinduction of GR mRNA (>50-fold after 6 h). Inhibition of transcription by AMD after 3 h of TPA treatment was associated with a decay of GR mRNA with a half-life of 2–3 h, which is identical to that observed in non-treated cells. We conclude that the increase in GR mRNA in the presence of TPA is dependent on ongoing transcription, whereas the rate by which GR transcripts are degraded, is not altered by TPA.

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INTRODUCTION

Glucocorticoids influence a great number of physiological processes [1]. The concentration of glucocorticoid receptors (GR) is a major factor determining the sensitivity of cells to glucocorticoids [2]. Numerous studies have shown that levels of GR are subjected to homologous regulation [3–10], as well as regulation by cyclic AMP [11–14] and other hormones and growth factors [15, 16]. The hormone binding capacity of GR is dependent on the phosphorylation state of the receptor protein [17, 18]. It is reported that the GR is phosphorylated in the presence of glucocorticoids [8, 19–21], cyclic AMP [11, 22], as well as epidermal growth factor [23]. Furthermore, mutual negative interference between protein kinase C (PKC)- and GR-dependent gene transcription is described in several systems [24, 25].

Calcium-dependent protein kinases, including PKC, mediate the effects of a wide variety of hormones, growth factors, and other biologically active substances [26–29]. Tumour-promoting phorbol esters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), have been shown to mimic the effects of diacylglycerol on activation of PKC [30]. Like diacylglycerol, TPA increases the affinity of PKC for calcium, resulting in full activation of this enzyme and phosphorylation of its specific substrate proteins. The major PKC subtype in MCF-7 cells requires both calcium and diacylglycerol/TPA to elicit full activation [31].

The MCF-7 cell line was derived from a human mammary adenocarcinoma [32]. These cells contain high levels of estrogen receptors (ER) [33], and is extensively used as a model system for studies on estrogen action. It has been demonstrated previously that the MCF-7 cells also express other steroid receptors, including GR [34]. The MCF-7 cells may therefore be a suitable model system for comparison of the two steroid receptors, ER and GR, with respect to hormonal regulation.

It is previously shown that TPA treatment of MCF-7 cells causes down-regulation of ER mRNA [35–38], and that this decrease in the level of ER

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Abbreviations: GR, glucocorticoid receptor, TPA, 12-*O*-tetradecanoylphorbol-13-acetate; AMD, actinomycin D; CHX, cycloheximide, PKC, protein kinase C; ER, estrogen receptor; cDNA; complementary DNA; kb, kilobases.

mRNA requires ongoing RNA synthesis but not protein synthesis [39]. The purpose of the present study was to investigate whether PKC activation by TPA might alter the level of GR as well as its mRNA in MCF-7 cells, and further try to elucidate possible mechanisms for such regulation. We show that treatment with TPA is associated with an increase in specific binding of [^{3}H]dexamethasone as well as a transient up-regulation of GR mRNA. Inhibitors of RNA synthesis prevent the TPA-dependent stimulation of GR mRNA, and the decay of GR mRNA is not altered by TPA. In contrast, inhibitors of protein synthesis increase the level of GR mRNA and dramatically amplify the TPA-dependent stimulation of GR mRNA.

EXPERIMENTAL

Chemicals

TPA (T 8139), actinomycin D (AMD) (A 4264), cycloheximide (CHX) (C 6255), and dexamethasone (D 1756) were supplied by Sigma (St Louis, MO). [α-^{32}P]dCTP (PB 10205; 3000 Ci/mmol) and [1,2,4,6,7-^{3}H]dexamethasone (TRK.645; 86 Ci/mmol) were purchased from Amersham Laboratories (Aylesbury, Bucks., England).

Culture conditions for MCF-7 cells

The human mammary carcinoma cell line (MCF-7 cells) was cultured as described previously [40]. The cells were plated at a density of 2×10^6 cells in 10-cm culture dishes (Costar, Cambridge, MA). Growth medium was minimum essential medium (041-01090, Gibco, Middlesex, England) supplemented with 5% fetal calf serum (Gibco 011-06290), Insulin Actrapid Human (1.7 μmol/l; Novo Industri A/S, Denmark), L-glutamine (2.0 mmol/l; Gibco 043-05030), penicillin (5×10^4 IU/l), streptomycin (50 mg/l), and fungizone (500 μg/l).

After 3 days in growth medium, incubations were continued in a serum-free defined medium for another 3 days before start of experiments. Defined medium was minimum essential medium supplemented with insulin, glutamine, and antibiotics/antifungal agents, as described above, in addition to 3.2% non-essential amino acids (Gibco 043-01140). Serum was substituted by triiodothyronine (T 2752; 0.196 μg/l), hydrocortisone (H 4001; 7.25 μg/l), vitamin B_{12} (V 2876; 1.36 μg/l), *d*-biotin (B 4501; 7.0 μg/l), *d,l*-α-tocopherol (T 3251; 10 mg/l), retinol (R 2750; 5.0 mg/l), *d,l*-6,8-thioctic acid (T 5625; 0.20 mg/l), linoleic acid (L 1376; 0.10 mg/l), and transferrin (T 4515; 1.0 mg/l), all obtained from Sigma.

Preparation and Northern blot analysis of RNA

Total RNA was extracted and analyzed by Northern blotting technique, essentially as described previously [40]. Each RNA sample was quantified (absorbance) in triplicate, and 20 μg/lane was resolved by gel electrophoresis. The RNA was visualized by staining with ethidium bromide before gel transfer to further evaluate RNA amount and quality. Autoradiography was performed for 6–14 days. The final autoradiographs were subjected to densitometric scanning in a Vitatron densitometer (Vitatron, Dieren, Holland), and signal intensities were calculated by a Hewlett-Packard integrator (Hewlett-Packard, Palo Alto, CA).

Complementary DNA (cDNA) probe and nick translation

The human GR cDNA was a 3.1 kilobase (kb) *Bam* HI fragment containing the complete open reading frame [41], kindly provided by Dr M. V. Govindan, Le Centre Hospitalier de l'Université Laval, Quebec, Canada. The GR cDNA was labeled with [α-^{32}P]dCTP by nick translation (Amersham kit No. N5000) according to the manufacturer's instructions, to give specific activities of $2–5 \times 10^8$ cpm/μg cDNA.

Preparation of soluble cell extracts

MCF-7 cells from 4 culture dishes were pooled and homogenized in ice-cold KTEDMo buffer [300 mM KCl, 10 mM Tris–HCl (pH 7.4 at 23°C), 1.5 mM EDTA (pH 7.4 at 23°C), 1 mM dithiothreitol, and 10 mM Na_2MoO_4], and cell extracts were prepared, essentially as described previously [40].

Steroid binding assay

GR levels were determined using a single concentration saturation assay containing 5 nM [^{3}H]dexamethasone with and without a 200-fold molar excess of unlabeled dexamethasone at 0°C for 14 h. Labeled cell extracts (100 μl) were incubated with 20 μl dextran-coated charcoal (2.5% charcoal and 0.25% dextran T-70 in KTEDMo buffer) at 0°C for 15 min. After centrifugation, 50–80 μl of the supernatants were removed and counted for tritium.

RESULTS

Induction of GR by TPA

MCF-7 cells were cultured in the presence of TPA (10^{-7} M) for 1–9 h, and levels of specific binding of [^{3}H]dexamethasone were measured. As seen from Fig. 1, TPA caused a time-dependent increase in [^{3}H]dexamethasone binding. The maximal level (34.8 ± 5.4 fmol/mg protein) was observed after 9 h of TPA treatment, and this was more than twice the level seen in untreated cells (16.0 ± 2.3 fmol/mg protein).

Biphasic regulation of GR mRNA by TPA

MCF-7 cells were treated with TPA (10^{-7} M) for 1–6 h, and levels of GR mRNA were analyzed by Northern blot hybridization. As seen from Fig. 2, both a major GR mRNA of 7.0 kb and a minor 6.5 kb mRNA [41] appeared to be subject to the same regulation. The major GR mRNA showed an initial increase (4- to 8-fold stimulation) after 2–3 h of TPA treatment, followed by a decline towards the control value after 6 h.

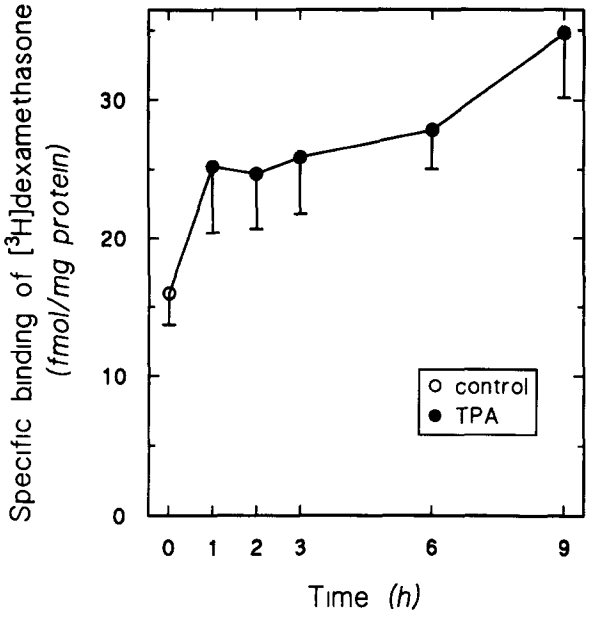

Fig. 1. Time-dependent changes in specific binding of [^{3}H]dexamethasone in the presence of TPA. MCF-7 cells were incubated in the presence of TPA (10^{-7} M) for the indicated time periods. Cells from 4 culture dishes were pooled, and soluble cell extracts were prepared. Levels of GR were measured using a single concentration saturation assay containing 5 nM [^{3}H]dexamethasone. GR levels are given as mean ± SD of triplicate determinations.

TPA (10^{-7} M) was the concentration required for maximal induction of GR mRNA (>5-fold) after 2 h of incubation of MCF-7 cells ($n = 3$; results not shown).

Requirement of ongoing transcription and translation for TPA-dependent increase in GR mRNA

MCF-7 cells were treated with TPA (10^{-7} M) and/or AMD (5.0 μg/ml) for 1–6 h, and levels of GR mRNA were investigated by Northern blot analysis. Figure 3 shows densitometric scanning values of the resulting GR mRNA signals (7.0 kb). In the presence of the RNA synthesis inhibitor AMD, GR mRNA showed a first-order decay (half-life 2–3 h). A very similar decay of GR mRNA was observed in cells cotreated with TPA and AMD, where AMD completely abolished the TPA-dependent increase in GR mRNA. Similar effects were also obtained with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (150 μM), an inhibitor of RNA polymerase ($n = 3$; results not shown).

Next, MCF-7 cells were incubated in the presence of TPA (10^{-7} M) and/or CHX (50 μM) for 1–6 h, and levels of GR mRNA were investigated by Northern blot analysis. Figure 4 shows densitometric scanning values of the resulting GR mRNA signals (7.0 kb). Treatment with CHX was associated with a time-dependent increase in the level of GR mRNA (>10-fold after 3–6 h). Coincubation of MCF-7 cells with TPA and CHX resulted in a superinduction in the level of GR mRNA (>50-fold after 6 h). Similar effects were also obtained with other inhibitors of protein synthesis [puromycin (50 μg/ml), anisomycin (10 μM)] ($n = 3$; results not shown).

Table 1 summarizes a number of experiments in which the effects of TPA and/or AMD or CHX on GR mRNA (the 7.0 kb transcripts) were examined.

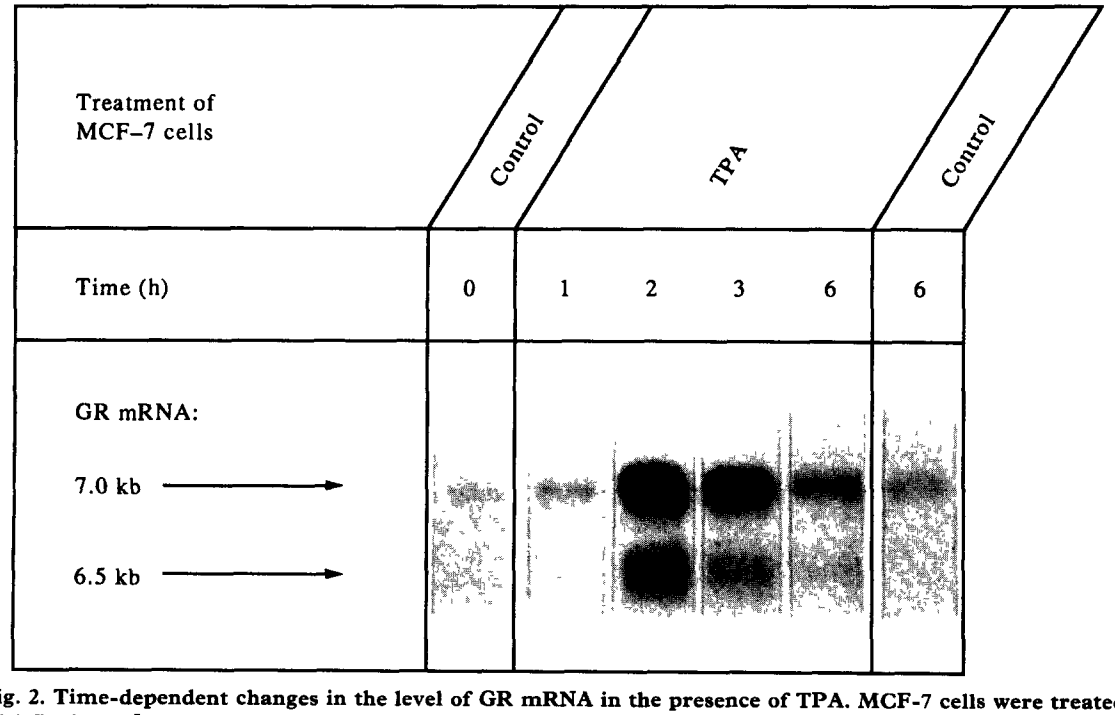

Fig. 2. Time-dependent changes in the level of GR mRNA in the presence of TPA. MCF-7 cells were treated with TPA (10^{-7} M) for the indicated time periods. Total RNA was prepared, and Northern blot analysis of GR mRNA was performed. The autoradiographs shown are representative for 12 independent experiments where both the major GR mRNA species of 7.0 kb and a minor 6.5 kb species [41] appeared.

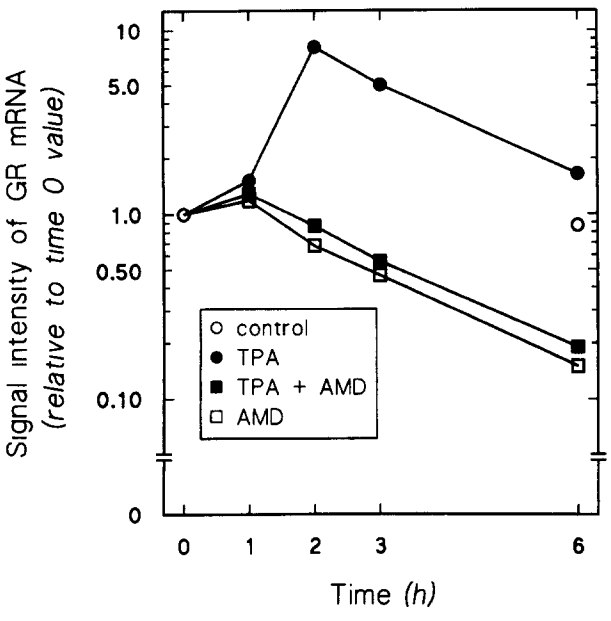

Fig. 3. Effect of AMD on TPA-dependent increase in GR mRNA. MCF-7 cells were incubated with TPA (10^{-7} M) and/or AMD [5.0 μg/ml (4.0 μM)] for 1–6 h, and levels of GR mRNA were analyzed by Northern blot hybridization. The figure represents densitometric scanning values of resulting GR mRNA signals (7.0 kb) representative for 3 independent experiments. Values are plotted relative to the time zero value.

Table 1. Requirement of inhibitors of transcription and translation for TPA-dependent increase in GR mRNA in MCF-7 cells

Treatment of MCF-7 cells	Signal intensity of GR mRNA (relative to control) Time 0 h	3 h	6 h
Control	1	—	—
TPA	—	5.2 ± 1.4[a]	2.4 ± 1.1[a]
TPA + AMD	—	0.53 ± 0.03[b]	0.27 ± 0.07[b]
AMD	—	0.47 ± 0.10[b]	0.28 ± 0.12[b]
TPA + CHX	—	24 ± 10[b]	63 ± 28[b]
CHX	—	17 ± 10[b]	10 ± 1.8[b]

MCF-7 cells were treated with TPA (10^{-7} M), a combination of TPA and AMD [5.0 μg/ml (4.0 μM)] or CHX (50 μM), or these inhibitors alone for 3 and 6 h, and levels of GR mRNA were analyzed by Northern blot hybridization. The data represent densitometric scanning values [relative to the time zero value (control)] of the resulting autoradiographs (the 7.0 kb GR mRNA signals), and are presented as mean ± SD of different number of experiments, [a]$n = 12$, [b]$n = 3$. Acid-precipitable [^{3}H]uridine incorporation of cell extracts was inhibited by 98–99% in the presence of AMD Acid-precipitable [^{14}C]leucine incorporation of cell extracts was inhibited by >90% in the presence of CHX

Effect of AMD on GR mRNA decay in the presence of TPA

Maximal induction of GR mRNA by TPA (between 4- and 8-fold) was observed between 2 and 3 h of incubation (Fig. 2). This was followed by a decline in the level of GR mRNA. To investigate the decay of GR mRNA in further detail, we analyzed half-lives of GR mRNA in control and TPA-treated cells.

Control cells and cells treated with TPA (10^{-7} M) for 3 h were further incubated for various time periods (1–6 h) in the presence of AMD (5.0 μg/ml). Incubation of MCF-7 cells with TPA alone was included for comparison. Levels of GR mRNA were analyzed by Northern blot analysis. Figure 5, which represents densitometric scanning values of the resulting GR mRNA signals (7.0 kb), shows that half-life of GR mRNA in control cells again was 2–3 h, confirming earlier observations (Fig. 3 and Table 1). Turnover of GR mRNA revealed identical, first-order decay whether TPA was present or not, indicating that the rate by which GR transcripts were degraded, was not altered by TPA.

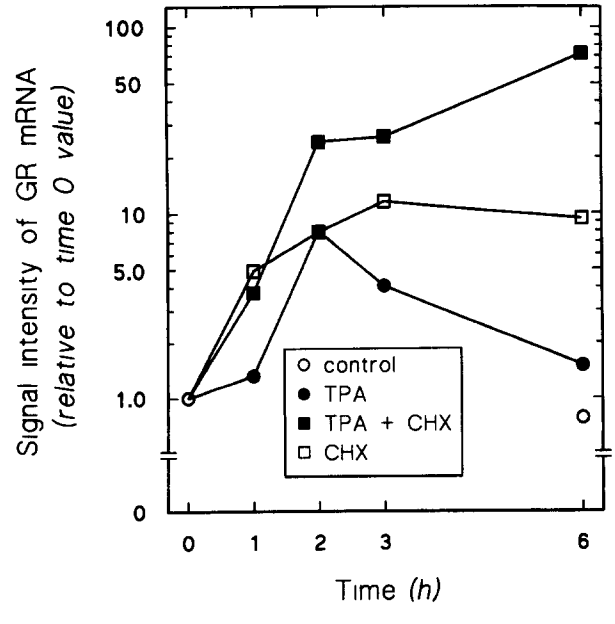

Fig. 4. Effect of CHX on TPA-dependent increase in GR mRNA. MCF-7 cells were incubated with TPA (10^{-7} M) and/or CHX (50 μM) for 1–6 h, and levels of GR mRNA were analyzed by Northern blot hybridization. The figure represents densitometric scanning values of resulting GR mRNA signals (7.0 kb) representative for 3 independent experiments. Values are plotted relative to the time zero value.

DISCUSSION

In this study we demonstrate that TPA treatment of MCF-7 cells is associated with an increase in specific binding of [^{3}H]dexamethasone (more than 2-fold) as well as a transient up-regulation of GR mRNA (4- to 8-fold). Inhibitors of RNA synthesis abolish the TPA-dependent increase in GR mRNA, indicating that the stimulatory effect of TPA is dependent on ongoing transcription. Furthermore, the decay of GR mRNA is identical whether TPA is present or not, indicating that the degradation rate of GR mRNA is not altered by TPA. In contrast, inhibitors of protein synthesis amplify the TPA-dependent stimulation of GR

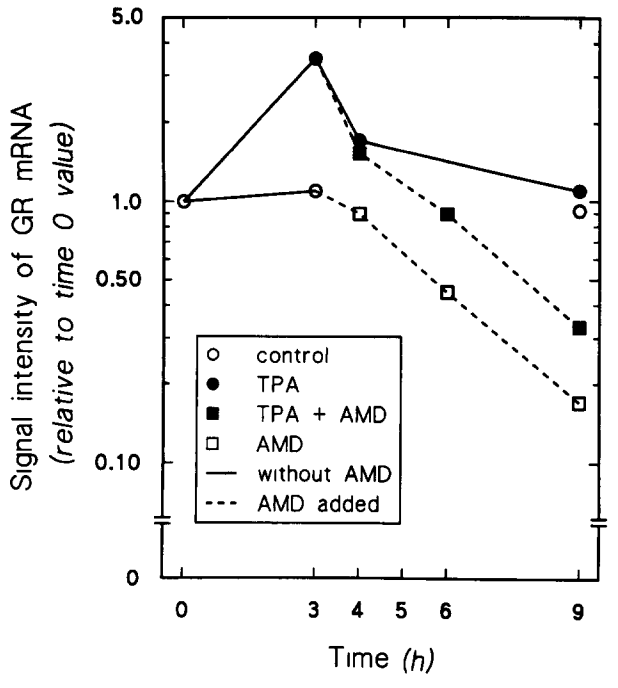

Fig. 5. Effect of AMD on GR mRNA decay in the presence of TPA. MCF-7 cells were treated with or without TPA (10^{-7} M) for 3 h. AMD [5.0 μg/ml (4.0 μM)] was then added to the cell cultures, and incubations were continued for additional 1–6 h. Incubations of MCF-7 cells with TPA alone were included for comparison. Levels of GR mRNA were analyzed by Northern blot hybridization, and the figure represents densitometric scanning values of resulting GR mRNA signals (7.0 kb) representative for 3 independent experiments. Values are plotted relative to the time zero value.

mRNA. The mechanism of superinduction may be interpreted as evidence for an inhibition of GR mRNA synthesis due to the loss of a short-lived repressor of transcription, and/or an increase in GR mRNA half-life due to the loss of a short-lived mRNA destabilizing factor.

Since we have previously examined TPA-dependent regulation of ER mRNA and protein levels in MCF-7 cells cultured in a standard defined medium containing hydrocortisone [36, 39], we decided to undertake the present study under conditions identical to those previously used. The data concerning specific binding of [^{3}H]dexamethasone (Fig. 1) may therefore not be entirely quantitative due to incomplete ligand exchange during the steroid binding assay. The levels of specific binding of [^{3}H]dexamethasone were, however, significantly higher than the control level at both 6 and 9 h of TPA treatment ($P \leqslant 0.01$; Student's t test).

It has been demonstrated that PKC activation by TPA leads to a rapid down-regulation of ER mRNA in the MCF-7 cells [35–38]. We have shown that this decrease is dependent on ongoing RNA synthesis but not protein synthesis, indicating that short-lived RNA molecules are involved in the rapid TPA-dependent down-regulation of ER mRNA [39]. Hence, treatment with TPA is associated with opposite regulatory effects on the mRNAs for ER and GR in MCF-7 cells.

Numerous studies have shown that GR mRNA and receptor protein are subjected to regulation by cyclic AMP [11–14] via stabilization of the mRNA for GR [13, 42], glucocorticoids [3–10], estradiol-17β [15], and epidermal growth factor [16]. Glucocorticoids generally down-regulate mRNA for their own receptors, reflecting decreased transcription rate of the GR gene [42–44], destabilization of the GR transcripts [45, 46], or both [47]. Furthermore, several studies suggest that TPA-induced activation of the transcription factor AP-1, which is a c-Jun homodimer or, preferably, a c-Jun/c-Fos heterodimer, antagonizes GR-dependent *trans*-activation by interacting with an overlapping *cis*-acting gene element [48] or by direct protein–protein interaction [49, 50].

The transient TPA-dependent stimulation of GR mRNA could be due to increased transcriptional activity of the GR gene promoter, enhanced processing of primary GR transcripts, or stabilization of mature cytoplasmic mRNAs [51]. In this study we show that TPA does not influence GR mRNA degradation. Attempts to study transcriptional activation of the GR gene using a "nuclear run on" transcription assay did not give conclusive results, due to very low levels of GR transcripts formed. So far we have not been in the position to study TPA-dependent regulation of nuclear processing of primary GR transcripts.

In several systems TPA has been shown to activate as well as repress transcriptional activity [52–55]. However, a consensus TPA response element [56, 57] in the 5′-flanking region of the GR gene has not been demonstrated. Studies on the 5′-flanking region of the GR gene [58–60] have revealed sequences responsible for glucocorticoid-dependent down-regulation [61, 62], more or less weak homologies for several cyclic AMP response elements and an estrogen response element, as well as an AP-1-like binding site [59]. This modified AP-1 binding site is the most obvious regulatory element to mediate transcriptional control by TPA. Although speculative; the cyclic AMP-like response elements may also confer TPA induction of GR promoter activity. TPA and cyclic AMP mediate their transcriptional responses through closely related *cis*-acting elements [56, 57, 63, 64], as well as common *trans*-acting factors [65, 66].

Taken together, these and previous results [35–39] suggest that PKC activation by TPA modulates gene regulation by steroids in MCF-7 cells. The down-regulation of ER mRNA in contrast to the stimulatory effect on GR mRNA, demonstrates that two steroid receptor systems are differentially regulated by TPA in these cells. At present, possible consequences of these opposite responses for cellular proliferation and differentiation, are not known.

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