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ARTICLES

Regulation of Prolactin Receptor Expression by the Tumour Promoting Phorbol Ester 12-*O*-Tetradecanoylphorbol-13-Acetate in Human Breast Cancer Cells

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Abstract In both the normal and malignant human breast, cellular sensitivity to the proliferative and differentiative activities of the lactogenic hormones is conferred by expression of the prolactin receptor (PRLR). The PRLR is regulated by steroid hormones; however, recent findings have suggested that PRLR may also be regulated by protein kinase C. To examine this possibility we have studied the effect of various modulators of PKC activity on PRLR binding activity and gene expression in five PRLR positive human breast cancer cell lines. Treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a tumour promoter and modulator of PKC activity, decreased PRLR binding activity in all cell lines examined. In MCF-7 cells, 10 nM TPA caused a 70% loss of PRLR mRNA after 12 h, paralleled 3 h later by a comparable loss of cell surface PRLR. Mezerein, a non-phorbol ester modulator of PKC activity and 1,2-dioctanoyl-*sn*-glycerol, a permeant analogue of the endogenous activator of PKC, also reduced PRLR binding activity and gene expression in a time- and concentration-dependent manner. Cycloheximide failed to abrogate the TPA-induced decline in PRLR mRNA levels, indicating that this process was not dependent upon continuing protein synthesis. No change in the stability of PRLR mRNA was observed during 24 h of TPA treatment and TPA reduced the rate of PRLR gene transcription within 3 h of treatment. These results demonstrate that modulators of PKC activity reduce PRLR binding activity and gene expression, implicating this signal transduction pathway in PRLR regulation.

Key words: prolactin receptor, phorbol ester, human breast cancer

Prolactin has many biological actions in vertebrates which include immunoregulation, osmoregulation, behaviour, reproduction, and the control of mammary gland development and lactation [Kelly et al., 1991]. In the mammary gland during pregnancy, prolactin and the sex steroid hormones stimulate proliferation of the ductal epithelium into the surrounding mammary stroma, to produce lobuloalveolar structures which are further stimulated by prolactin post-partum, resulting in lactation [Vonderhaar, 1987]. The mitogenic activity of prolactin is also observed in rodent mammary tumours and human breast cancers, where prolactin stimulates cell growth [Vonderhaar and Biswas, 1987]. Thus prolactin plays a role in the etiology of human breast cancer that may be analogous to the activity of prolactin in the normal breast.

Cellular sensitivity to prolactin is conferred by expression of the prolactin receptor (PRLR), which in humans binds human growth hormone, human placental lactogen, and prolactin [Ormandy et al., 1990]. Recent studies have shown that the PRLR is a transmembrane glycoprotein of 598 amino acids with sequence homology to the receptors for growth hormone, the interleukins, and erythropoietin. The PRLR has a wide tissue distribution, consistent with the range of prolactin's biological effects [Kelly et al., 1991]. The role of prolactin in the control of mitogenesis in normal and malignant breast makes an understanding of the regulation of PRLR expression fundamental to the study of

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normal breast development and the etiology of breast cancer. In human breast cancer cells and rodent mammary gland the expression of the PRLR is regulated by steroid hormones, differentiation agents, and prolactin [Ormandy and Sutherland, in press].

Several recent observations suggest that the multi-gene protein kinase C (PKC) family [Nishizuka, 1988] may also control PRLR expression. PKC has been implicated in PRLR signal transduction [Rillema et al., 1988] which suggests that prolactin regulation of PRLR levels may occur via a PKC-mediated mechanism. In addition the differentiation agent and modulator of PKC activity, 12-*O*-tetradecanoyl-13-acetate (TPA) [Jaken, 1990], decreased estrogen receptor in MCF-7 cells [Guilbaud et al., 1988; Lee et al., 1989]. Since PRLR and estrogen receptor are co-expressed in breast tumours and are co-regulated by differentiation agents [Ormandy and Sutherland, in press], TPA may also decrease PRLR expression.

To investigate whether PKC is involved in the regulation of PRLR gene expression, the effect of various modulators of PKC activity on PRLR expression was examined using 5 PRLR+ human breast cancer cell lines.

MATERIALS AND METHODS

Materials

Actinomycin D, cycloheximide, 1,2-dioctanoyl-*sn*-glycerol, mezerein, and the phorbol esters 4α-phorbol-12,13-didecanoate and 12-*O*-tetradecanoylphorbol-13-acetate were from the Sigma Chemical Co. (St. Louis, MO). Human growth hormone, the most potent ligand for the human PRLR [Ormandy et al., 1990], was kindly donated by Dr. G. Chapman. ^{125}Iodine and α[^{32}P]-deoxycytidinetriphosphate were from Amersham (Sydney, N.S.W., Australia). The PRLR cDNA was the H1/H2 construct covering the entire coding region [Boutin et al., 1989]. The oligonucleotide to the 18S ribosomal subunit was synthesized to bases 151–180 of the rat sequence [Chan et al., 1984]. Cells were from E.G. and G. Mason Research Institute (Worcester, MA), (MCF-7, T-47D) and the American Type Culture Collection (Rockville, MA) (BT-474, MDA-MB-453, ZR-75-1). Cell culture reagents were from C.S.L.-Novo (Sydney, N.S.W., Australia). Tissue culture plates were from Linbro (Sydney, N.S.W., Australia), and flasks were from Corning (Sydney, N.S.W., Australia). All other reagents were of Molecular Biology or AR grade from Sigma and Bio-Rad (Sydney, N.S.W., Australia).

Cell Culture

Cell lines were passaged as previously described [Reddel et al., 1985] in RPMI-1640 medium supplemented with 6 mM L-glutamine, 10 μg/ml porcine insulin, 20 μg/ml gentamycin sulphate, and 5% fetal calf serum (RPMI–5% FCS). For the assay of PRLR binding activity, cells were plated from exponential growth phase cultures into 12 well tissue culture plates at 1×10^5 cells in 2 ml of RPMI–5% FCS per well and incubated in 5% CO_2-air at 37°C until confluent. The medium was replaced with fresh RPMI–5% FCS containing various TPA concentrations added from 1000× Me_2SO stock solutions. For Northern analysis and assay of transcription rate, 1×10^6 exponentially growing cells were plated in 50 ml of RPMI–5% FCS per 150 cm^2 flask and grown to confluence. TPA and mezerein were added directly from 1000× stock solutions in Me_2SO. DiC8 was added from 1000× stock solution in Me_2SO to 20 μg/ml every 30 min for 12 h.

Assay of PRLR Binding Activity

Wells were drained and assayed using 60,000 c.p.m./well of iodinated human growth hormone (hGH) as the radioligand. Wells were incubated at room temperature overnight. Non-specific binding was estimated by inclusion of 45 nM unlabelled hGH. Cell numbers were determined in parallel wells with a hemocytometer. The specific binding standard error was calculated by taking the square root of the sum of the squares of the total and non-specific binding standard errors.

Northern Analysis

Flasks were drained, followed by the addition of 7 ml of guanidinium isothiocyanate solution and shearing of the DNA by repeated passage of the lysate through a 21 gauge needle. Total cellular RNA was prepared and PRLR mRNA levels were measured as previously described [Lee et al., 1989], in 20 μg/lane of total RNA using a [^{32}P]deoxycytidinetriphosphate random

primer labelled PRLR cDNA. Variation in RNA content per lane was measured by reprobing with an oligonucleotide for the 18S ribosomal subunit. Autoradiographs were quantified by longitudinal densitometric scans and were analyzed using the Bio-Rad 1D analyst computer programme.

Assay of PRLR Transcription Rate

The rate of transcription of the PRLR gene was measured using 4×10^7 cells per point by the method of Greenberg and Ziff [1984] with modifications as previously described [Clarke et al., 1991]. Briefly, intact nuclei isolated at various times after the addition of TPA or vehicle were allowed to continue transcription for 30 min in the presence of 200 μCi of α[^{32}P]uridine-triphosphate, prior to isolation of total RNA. A 10 μl sample from each pool of labelled RNA was counted and the specific activities of each treatment group were normalized accordingly by dilution, prior to hybridization of equal volumes with 5 μg of PRLR cDNA (immobilized on nitrocellulose) for 72 h. Filters were then washed and autoradiographed for 4 weeks with two intensifying screens at −70°. pUC-12 DNA was used as a negative control. Results were expressed as a percentage of the time-matched, vehicle-treated controls.

Assay of PRLR mRNA Half-Life

Cells were treated with 10 nM TPA or vehicle for various periods of time up to 24 h. After each of these treatment periods transcription was stopped by the addition of actinomycin D and the time course of the resulting decline in PRLR mRNA levels was measured by Northern analysis at 0, 1, 2, 4, 6, and 12 h, to determine whether TPA treatment resulted in a faster rate of PRLR mRNA degradation. Autoradiographs were quantified by measurement of optical density and the data was expressed as a percentage of the 0 h control. Variations in loading were measured by reprobing for 18S rRNA. To calculate PRLR mRNA half-life, decay curves were linearized by $\log_{10}$ transformation of the optical density values and linear regression was used to calculate the line of best fit. Half-life was calculated from the equation of the line of best fit as the time taken for a 50% decline in PRLR mRNA level.

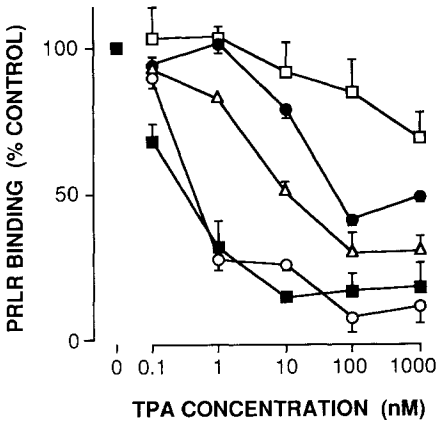

Fig. 1. Effect of TPA on PRLR binding activity in human breast cancer cell lines. Cells were grown in RPMI 1640–5% FCS in 12 well plates. When confluent they were treated in triplicate with various concentrations of TPA or Me_2SO vehicle for 24 h, followed by assay of PRLR binding activity using iodinated human growth hormone as detailed in Materials and Methods. Specific binding varied between 500 and 2,000 c.p.m. per well. Results are expressed as a percentage of vehicle-treated control and error bars represent the standard error of the mean. □, ZR-75-1; ●, BT-474; △, T-47D; ○, MDA-MB-453; ■, MCF-7.

RESULTS

Effect of TPA Treatment on PRLR Binding Activity and mRNA Levels in Human Breast Cancer Cells

Five human breast cancer cell lines known to express PRLR were treated with various concentrations of TPA for 24 h and then assayed for PRLR binding activity (Fig. 1). A decrease in PRLR binding activity occurred in all cell lines and sensitivity to TPA varied between cell lines. The MCF-7 cell line was the most sensitive as the concentration of TPA which reduced the binding by 50% was 0.3 nM, followed by MDA-MB-453 (0.5 nM), T-47D (10 nM), BT-474 (100 nM), and ZR-75-1 (> 1,000 nM). Further experiments were conducted with the MCF-7 cell line. The time course of changes in PRLR mRNA and binding activity were compared. Northern blot analysis of total cellular RNA using the H1/H2 cDNA detected 4 major PRLR transcripts of 10.5, 8.6, 3.5, and 2.7 kb relative to the ribosomal subunits (Fig. 2). These transcripts were also seen using poly-A$^+$ RNA (data not shown). TPA treatment resulted in an apparently equal decrease in all four mRNA species. PRLR mRNA levels fell to 60% of control after 3 h and reached a minimum of 30% of control after 12 h (Fig. 3). PRLR binding activity followed a similar time course but with a delay of approximately 3 h, suggesting that the loss of PRLR mRNA was the

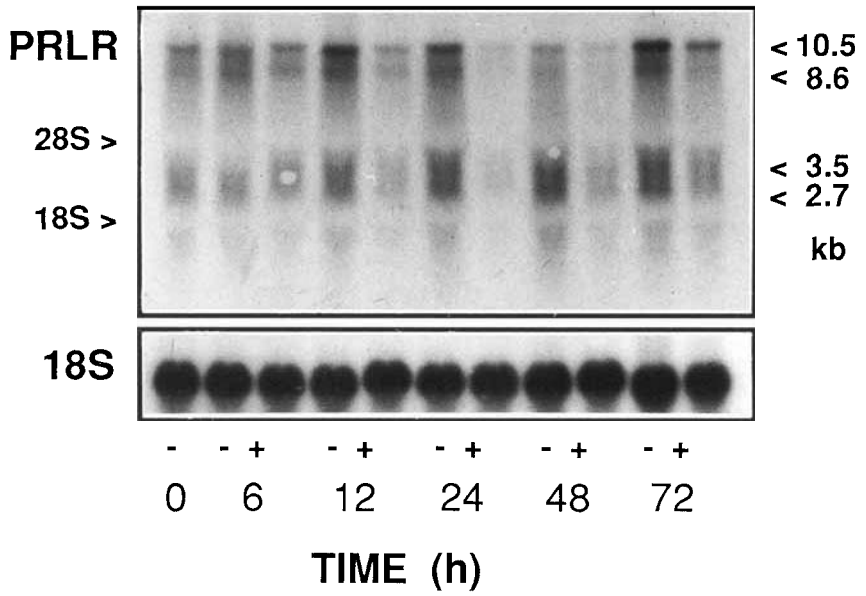

Fig. 2. Northern analysis of the effect of TPA on PRLR mRNA levels in MCF-7 human breast cancer cells MCF-7 cells were treated with 10 nM TPA (+) or vehicle (−) for the indicated times prior to measurement of PRLR mRNA levels by Northern analysis as described in Materials and Methods The autoradiograph was exposed for 2 days without intensifying screens Arrows on the left indicate the position of the 28S and 18S ribosomal subunits Arrows on the right indicate the size and position of the 4 PRLR mRNA species detected Variations in RNA loading between lanes were measured by reprobing with an oligonucleotide to the 18S ribosomal subunit **(lower panel)** The autoradiograph was exposed for 3 h without intensifying screens

primary cause of the decline in binding activity. At 48 and 72 h, PRLR mRNA levels increased from their nadir at 24 h to around 50% and 60% of control, respectively, followed by a similar increase in binding activity approximately 24 h later. The phorbol ester 4-α-phorbol-12,13-didecanoate, an inactive tumour promoter and PKC modulator [Darbon et al., 1986], had no effect on PRLR mRNA levels (data not shown). Scatchard analysis [Scatchard, 1949] demonstrated that the TPA-induced loss of binding activity was due a loss of binding sites (Fig. 3 inset) and not to a change in receptor affinity, as expected from the observed parallel changes in PRLR binding activity and mRNA (Fig. 3). MCF-7 PRLR had a dissociation constant of 0.48 nM for human growth hormone and expressed 5,100 PRLR molecules per cell.

Effect of Treatment With DiC8 and Mezerein on PRLR mRNA and Binding Activity in MCF-7 Cells

To provide further evidence for PKC mediated regulation of PRLR gene expression, MCF-7 cells were treated with other modulators of PKC activity and the effect on PRLR mRNA and binding activity was determined. Treatment with 10 nM mezerein, a diterpene tumour promoter, produced a time-dependent decrease in PRLR mRNA levels to 40% of vehicle-treated control cells after 24 h (Fig. 4A). Mezerein also produced a concentration-dependent decrease in PRLR binding activity to a minimum of around 30% of control after 24 h exposure to concentrations of 10 nM and greater (Fig. 4B). When MCF-7 cells were treated with DiC8, a permeant analogue of the endogenous PKC activator diacylglycerol, a time-dependent decrease in PRLR binding activity was observed (Fig. 4C). Taken together, these observations show that three structurally diverse modulators of PKC activity have a similar effect on PRLR expression, strongly implicating PKC as a mediator of PRLR gene expression.

Effect of Cycloheximide on the TPA-Induced Reduction of PRLR mRNA

To investigate whether the mechanism by which TPA reduced PRLR mRNA involved the synthesis of new proteins, MCF-7 cells were treated with 10 nM TPA in the presence and absence of 20 μg/ml of cycloheximide for 3 and 6 h (Fig. 5). TPA alone reduced PRLR mRNA to

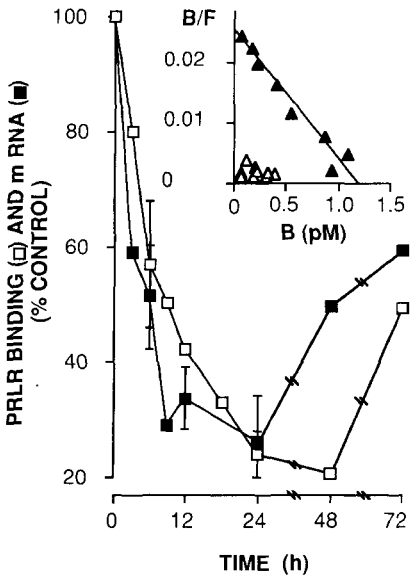

Fig. 3. Effect of TPA on PRLR binding activity and mRNA levels in MCF-7 human breast cancer cells. MCF-7 cells were treated with 10 nM TPA for various times prior to measurement of PRLR binding activity using iodinated human growth hormone (□) or extraction of total RNA and measurement of PRLR mRNA by Northern analysis (■). Results are expressed as a percentage of vehicle-treated, time-matched control cells. Error bars (at 6, 12, and 24 h for PRLR mRNA) represent the standard error of the mean of three separate experiments. Points without error bars are derived from either one or two experiments. **Inset:** Scatchard analysis [Scatchard, 1949] of PRLR binding activity from triplicate determinations after treatment with either 10 nM TPA (△) or Me_2SO vehicle (▲) for 24 h.

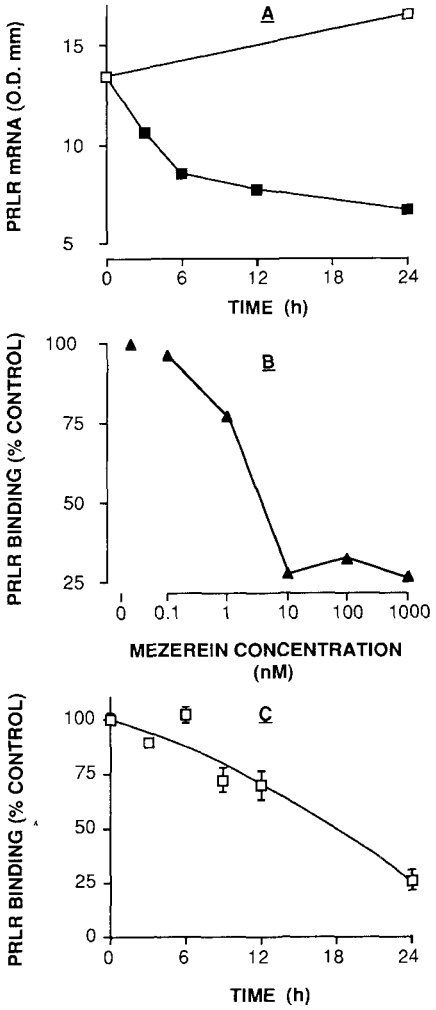

Fig. 4. Effect of mezerein and 1,2-dioctanoyl-*sn*-glycerol on PRLR binding activity and mRNA levels. **A:** MCF-7 cells were treated with vehicle (□) or 10 nM mezerein (■) for various times prior to measurement of PRLR mRNA by Northern analysis. **B:** MCF-7 cells were treated with various concentrations of mezerein for 24 h prior to assay of PRLR binding activity. Results are expressed as a percentage of time-matched, vehicle-treated controls. **C:** MCF-7 cells were treated with 20 μg/ml of 1,2-dioctanoyl-*sn*-glycerol every 30 min for various times up to 12 h prior to assay of PRLR binding activity. Results are expressed as a percentage of time-matched, vehicle-treated cells.

60% of control after 3 h and to 46% of control after 6 h. Cycloheximide alone had little or no effect on PRLR mRNA levels. Cycloheximide in combination with TPA failed to abrogate the effect of TPA at both times, indicating that the TPA-induced decrease in PRLR mRNA did not require continuing protein synthesis.

Effect of TPA on PRLR mRNA Half-Life and the Rate of PRLR Gene Transcription

The fall in PRLR mRNA produced by TPA treatment may be due to either an increase in the rate of PRLR mRNA degradation or a decrease in the rate of PRLR gene transcription. To examine whether TPA had an effect on PRLR mRNA half-life, MCF-7 cells were treated with 10 nM TPA or vehicle for either 0, 3, 6, 12, or 24 h prior to the addition of the transcription inhibitor actinomycin-D (5 μg/ml) and the measurement of the rate of PRLR mRNA decay by Northern analysis. There was no significant difference in the mRNA decay curves between TPA- and vehicle-treated cells when actinomycin D was added simultaneously (Fig. 6A). To examine whether a destabilization of PRLR mRNA may have occurred at later times during the 24 h period over which PRLR mRNA levels fell in response to TPA, MCF-7 cells were treated with TPA for various times prior to measurement of PRLR mRNA half-life using actinomycin D (Table I). No change in half-life was detected at any time and over all no significant difference in

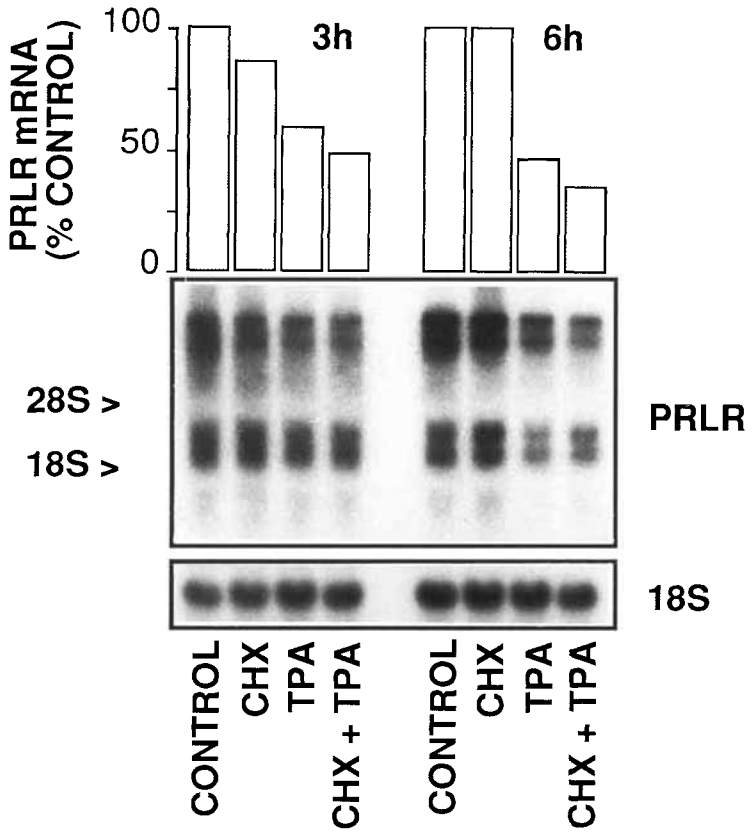

Fig. 5. Effect of cycloheximide on the reduction of PRLR mRNA levels by TPA. MCF-7 cells were treated with vehicle (CONTROL), 20 μg/ml of cycloheximide (CHX), 10 nM TPA (TPA), and 20 μg/ml cycloheximide with 10 nM TPA (CHX + TPA) for 3 and 6 h prior to measurement of PRLR mRNA by Northern analysis. The autoradiograph was exposed for 5 days without intensifying screens and quantified by densitometry. Results were expressed as a percentage of the time-matched, vehicle-treated control and are shown as histograms above the autoradiograph. The variation in RNA loading between lanes was measured by reprobing with an oligonucleotide to the 18S ribosomal subunit.

half-life was found between vehicle- and TPA-treated cells using a paired two-tail Student's t-test ($P = 0.68$). The average PRLR half-life was calculated as 6.4 h. Thus at no time during 24 h of TPA treatment was the TPA-induced decrease in PRLR mRNA due to increased rates of PRLR mRNA degradation.

The effect of TPA on the rate of PRLR gene transcription was investigated using nuclear run-on assays (Fig. 6B). After treatment of MCF-7 cells with 10 nM TPA the rate of PRLR gene transcription fell to 68% of control after 1 h, to 41% of control at 3 h, and then increased slightly to 59% of control at 6 h. No signal was detected from the pUC-12 slots, indicating negligible non-specific binding of the labelled RNA transcripts. In addition, the transcription rate of *c-myc* and the epidermal growth factor receptor was increased at these times (data not shown), indicating that TPA did not cause a general non-specific decrease in the rate of transcription. These data demonstrated that TPA treatment reduced the rate of PRLR gene transcription.

DISCUSSION

In human breast cancer cells TPA exerts a range of effects which include reduced cell proliferation rate and altered cellular morphology accompanying a more differentiated phenotype [Osborne et al., 1981], and reduced estrogen receptor binding activity and gene expression [Guilbaud et al., 1988; Lee et al., 1989]. The effects of TPA are thought to be mediated via modulation of PKC activity [Jaken, 1990]. Within the first hour of treatment, TPA increased PKC-mediated protein phosphorylation in human breast cancer cells [Issandou et al., 1986] and caused translocation of cytosolic PKC to the membrane fraction [Darbon et al., 1986],

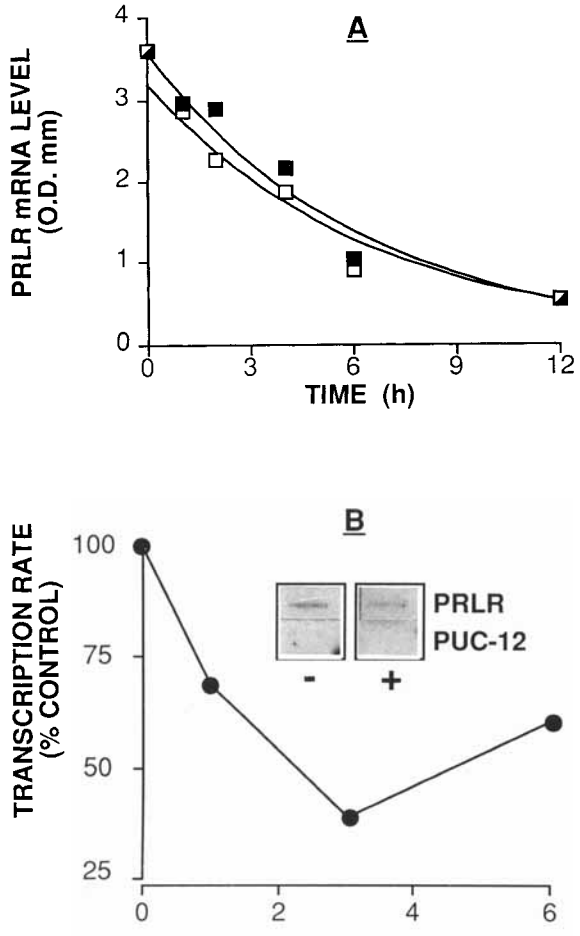

Fig. 6. Effect of TPA on PRLR mRNA half-life and the rate of PRLR gene transcription. **A:** MCF-7 cells were simultaneously treated with 10 nM TPA and 5 μg/ml actinomycin D (■) or actinomycin D alone (□) prior to harvest and measurement of PRLR mRNA by Northern analysis at the indicated times. Logarithmic lines of best-fit shown have r^2 values of 0.95 (■) and 0.92 (□) and are not significantly different from each other. **B:** MCF-7 cells (4×10^7 cells/point) were treated in duplicate with 10 nM TPA or Me_2SO vehicle for 1, 3, and 6 h prior to cell harvest and isolation of intact nuclei. Run-on RNA transcripts were labeled by incubation with α[^{32}P]UTP for 30 min and were isolated using PRLR cDNA immobilized on nitrocellulose filters. Transcription rates for PRLR (●) are expressed as a percentage of time-matched, vehicle-treated controls. **Inset:** Photographs of one of duplicate slots for PRLR and the PUC-12 negative control after 3 h exposure to TPA (+) or vehicle (−). Autoradiographs were exposed for 1 month with 2 intensifying screens and were quantified by densitometry.

prior to depleting the PKC protein and PKC activity from both the cytosolic and membrane fractions after 6–12 h [Darbon et al., 1986]. However, some activity remained [Issandou et al., 1988] and inactive PKC accumulated in the membrane fraction [Borner et al., 1988]. Immunoblot analysis showed that MCF-7 cells expressed PKC-α and PKC-τ but not PKC-β_{II} and that TPA caused the transfer of only PKC-α to the membrane fraction [Kennedy et al., 1992]. Thus in breast cancer cells two effects of TPA occur: initial activation of PKC activity followed by cellular depletion of specific PKC isoforms.

TABLE I. Effect of TPA Treatment on PRLR mRNA Half-Life*

Time (h)	PRLR mRNA HALF-LIFE (h)[a]			
	+ TPA	r[b]	− TPA	r[b]
0	4.3	0.94	3.8	0.93
3	5.7	0.92	8.2	0.96
6	4.7	0.84	4.7	0.99
12	8.0	0.97	5.8	0.95
24	7.2	0.85	9.3	0.89
Mean	6.0 ± 0.7 (n = 5)		6.4 ± 1.0 (n = 5)	

*MCF-7M cells were treated with TPA (+ TPA) or vehicle (− TPA) for the indicated times prior to determination of PRLR mRNA half-life using actinomycin D induced transcriptional inhibition and assay of the resulting decline in PRLR mRNA by Northern analysis.
[a]Determined as described in Materials and Methods.
[b]Correlation coefficient of the line used to calculate half-life.

To investigate the role of PKC in the regulation of PRLR gene expression, human breast cancer cell lines were treated with TPA. Initial investigation using five human breast cancer cell lines showed that TPA reduced PRLR binding activity in a time- and concentration-dependent manner in all PRLR+ cell lines. This observation demonstrated the general nature of this effect on the PRLR and indicated that the order of sensitivity of PRLR levels to TPA followed that previously reported for the ability of TPA to decrease cell number and epidermal growth factor receptors [Koga et al., 1990]. This result is also consistent with the observation that TPA reduced the cortisol-induced increase in prolactin binding to mouse mammary cells after 3 days of exposure [Taketani and Oka, 1983]. Scatchard analysis showed that the decrease in PRLR binding activity was due to a loss of receptor sites and Northern analysis indicated that this loss was due to decreased PRLR mRNA levels. A slight rise in PRLR mRNA levels occurred in control cells with time and may represent a response to conditioned media, cell-cell contact, or cell-extracellular matrix contact, all of which increase with time. These effects of TPA suggested that the PKC signal transduction pathway is involved in the regulation of PRLR expression.

TPA may, however, exert some non-PKC mediated effects. For example, recent reports have identified a number of proteins with consensus phorbol ester binding sequences but only one of these (n-chimaerin) has been shown to bind phorbol ester [Ahmed et al., 1990, and references therein; Coppola et al., 1991]. In addition the presence of a steroid hormone receptor-like TPA binding protein in human cells has been demonstrated [Hashimoto and Shudo, 1991]. Other investigators hypothesize that tumour promoters may operate via stimulation of arachidonic acid metabolism [Watanabe et al., 1990]. To provide further evidence for the involvement of PKC in the regulation of PRLR expression, the effects of modulators of PKC activity which differ from TPA in chemical structure and tumour promoting activity were examined. DiC8 is a permeant analogue of the endogenous activator of protein kinase C [Jaken, 1990] and is a member of the sn-1,2-diacylglycerol family, members of which are classified as stage II or complete tumour promoters, depending upon dosing regimes [Smart et al., 1989]. Previous investigations using MCF-7 cells showed that a single 40 μg/ml addition of DiC8 resulted in PKC mediated phosphorylation of an endogenous 28K protein [Issandou et al., 1988], accompanied by a transient reduction in cytosolic PKC activity [Issandou and Darbon, 1988]. The rapid metabolism of DiC8 necessitates repeated addition of this compound. The diterpene mezerein is a stage II tumour promoter which both activates [Miyake et al., 1984] and depletes [Grove and Mastro, 1991] PKC activity, analogous to the effect of TPA. DiC8 caused a time-dependent decrease in PRLR binding whilst mezerein caused a time- and concentration-dependent decrease in PRLR mRNA and binding activity. The time course for the fall in PRLR mRNA was similar for all three reagents tested. These experiments provide strong evidence to support the hypothesis that PKC is involved in the regulation of PRLR gene expression.

Alterations in mRNA levels can occur due to changes in mRNA stability or the rate of gene transcription. Investigation of PRLR mRNA stability by inhibition of transcription with actinomycin D showed no alteration in PRLR mRNA half-life at any time during 24 h of treatment with TPA. The measured 6.4 h half-life of PRLR mRNA is likely to be an overestimate due to the inhibition by actinomycin D of the transcription of rapidly degraded proteins normally involved in mRNA turnover [Mullner and Kuhn, 1988]. The data shown in Figure 3 suggests a half-life of around 3 h for PRLR mRNA, as PRLR transcription continued at a reduced rate during TPA treatment. These results suggested that TPA exerted an effect on the rate of PRLR transcription. Nuclear run-on assays demonstrated that TPA initiated a decrease in the rate of PRLR gene transcription within 60 min of addition, which therefore preceded the decline in PRLR mRNA levels and the depletion of PKC by TPA (discussed above), which suggested that TPA stimulation of PKC activity resulted in reduced PRLR gene expression. Investigation of PKC levels in human breast cancer cell lines has shown an inverse correlation between the level of PKC expression (measured by immunoblots, PKC activity, and phorbol ester binding) and estrogen receptor expression [Borner et al., 1987]. Since the PRLR and estrogen receptor are co-expressed in breast cancer cells [Ormandy and Sutherland, in press], higher levels of PKC activity are likely to occur in cells which have low levels of PRLR, consistent with the hypothesis that increased PKC activity results in reduced levels of PRLR gene expression and binding activity in human breast cancer cells.

Signal transduction via PKC is known to influence gene transcription rates via modulation of the activity of a variety of transcription factors including AP-1 and NF-κB [Chiu et al., 1987; Jones et al., 1988]. Cycloheximide, an inhibitor of protein synthesis, failed to abrogate the effect of TPA on PRLR mRNA levels, which indicated that TPA did not require continuing protein synthesis to reduce PRLR gene expression. In the light of the transcriptional nature of the TPA effect on PRLR mRNA, this result suggests that transcription factors which are regulated solely via the induction of gene expression are not involved in the TPA regulation of PRLR expression. Transcription factors which are regulated via phosphorylation/dephosphorylation processes such as the AP-1 and NF-κB transcription complexes [Hunter and Karin, 1992], provide examples of the downstream targets of PKC that may be involved in TPA regulation of PRLR levels.

Taken together these data suggest that TPA modulation of the level of the PRLR protein occurs via transcriptional regulation of PRLR gene expression and implicate a signal transduc-

tion pathway utilizing PKC mediated phosphorylation of pre-existing proteins in this process.

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