Evidence for the Involvement of Protein Kinase C in the Inhibition of Prolactin Gene Expression by Transforming Growth Factor-β2

CHUAN-CHANG CHUANG, SAI-KOONG TAN, LUNG-KUO TAI, JIN-PING HSIN, and FUNG-FANG WANG

Institute of Biochemistry (C.-C.C., S.-K.T., L.-K.T., J.-P.H., F.-F.W.) and Faculty of Medical Technology (S.-K.T.), National Yang-Ming University, Shih-Pai, Taipei, Taiwan 11221

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ABSTRACT

We investigated the mechanisms by which transforming growth factor (TGF)-β2 inhibited prolactin mRNA expression in GH3 rat pituitary tumor cells. Maximal inhibition was observed with cells exposed to 5 ng/ml TGF-β2 for 24 hr. Continuous presence of the hormone during the entire period was not necessary because exposure of cells to TGF-β2 for 20 min was sufficient to trigger the same extent of prolactin mRNA inhibition at 24 hr as with its persistent presence. The action of TGF-β2 could be abolished by cycloheximide or EGTA, suggesting the requirement of a newly synthesized protein and extracellular Ca2+. The response of prolactin mRNA to TGF-β2 was inhibited by preincubation of cells with phorbol-12-myristate-13-acetate, which down-regulated protein kinase C (PKC). The activities of both the cytosolic and membrane PKC were significantly reduced at 20 min after TGF-β2 addition, and inhibition continued to 24 hr, the last time point analyzed. However, the ratio of cytosolic to membrane PKC was not altered by TGF-β2. Inhibition of PKC did not require the sustained presence of TGF-β2. In vitro kinase assays of the immunoprecipitated PKC demonstrated that the activities of α, ε, μ, and ζ isozymes were significantly decreased in the TGF-β2-treated cells, whereas that of PKCλ was not affected. Western blotting did not reveal any change in PKCε steady state protein levels, suggesting TGF-β2 inhibits PKC activity through a post-translational mechanism. Our results support that inhibition of PKC activity is an early event mediating TGF-β2-inhibited prolactin mRNA expression in GH3 cells.

Prolactin is a lactotropic polypeptide hormone originally found to be secreted by the pituitary. Recent studies indicate that thyocytes (Montgomery et al., 1992) and lymphocytes (Pellegrini et al., 1992) also express and release prolactin. The prolactin receptor belongs to the cytokine receptor superfamily and is present in B and T lymphocytes and macrophages (Gagnerault et al., 1993). These findings have led to the suggestion of a novel role for prolactin as a cytokine capable of stimulating immune responses. The secretion and synthesis of prolactin in the pituitary are regulated by neurotransmitters and neuropeptides. For example, estrogen (Maurer, 1981) and TRH (Albert and Tashjian, 1984) have been shown to stimulate prolactin secretion or gene expression, whereas dopamine (Ben-Jonathan, 1985) and somatostatin (Lamberts et al., 1989) negatively regulate prolactin release. These factors act through a variety of signaling pathways to modulate the concentrations of this physiologically important hormone.

TGF-β is composed of a family of 25-kDa multifunctional proteins that play significant roles in a wide range of biological activities, including cell growth, differentiation, development, and gene expression (Massague et al., 1994). Three distinct TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β3, have been identified in mammals. These proteins are encoded by a family of closely related genes, and each exhibits similar but not identical functions. TGF-β isoforms are differentially distributed in various tissues and cell types. Recent evidence indicates that lactotrophs are TGF-β immunopositive, and the production of TGF-β in these cells can be negatively influenced by estrogen (Pastorcic et al., 1995). Moreover, expression of type II receptors for TGF-β has been demonstrated in lactotrophs (De et al., 1996). These findings suggest that TGF-β may act via a paracrine/autocrine mechanism in regulating normal pituitary function. Evidence reveals that TGF-β exerts an inhibitory action on the basal

ABBREVIATIONS: TGF, transforming growth factor; PMA, phorbol-12-myristate-13-acetate; PKC, protein kinase C; TRH, thyrotropin releasing hormone; MAP, mitogen-activated protein kinase; MBP, myelin basic protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GH, growth hormone; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.
and TRH-stimulated prolactin secretion in cultured anterior pituitary cells (Minami and Sarkar, 1997; Tan et al., 1997). In addition, Delidow et al. (1991) have shown that TGF-β inhibits both the basal and Ca2+-stimulated prolactin mRNA levels through transcriptional regulation in GH3 pituitary tumor cells.

A number of receptors for TGF-β have been cloned, and its mode of action has been elucidated (Miyazono et al., 1994). Extensive work demonstrates that TGF-β activates divergent signaling components in many distinct cell types (Derynck and Feng, 1997). In several epithelial cell lines, TGF-β activates Ras and increases the activity of MAP kinase (Yan et al., 1994). TAK-1, a member of the MAP kinase kinase family, was recently shown to be activated by TGF-β (Yamaguchi et al., 1995). In MvILu cells, TGF-β induces the phosphorylation of the cAMP response element binding protein (Kramer et al., 1991). In Rat-1 cells, treatment of TGF-β is accompanied by increased phosphatidylinositol turnover (Muldoon et al., 1995). Halstead et al. (1995) have shown that PKC and phosphatidylcholine phospholipase C may be involved in the TGF-β signaling that leads to elevated gene expression. Calcium has also been implicated in TGF-β signaling. In both rat Sertoli cells (Grasso et al., 1993) and Rat-1 fibroblasts (Muldoon et al., 1988), TGF-β stimulates Ca2+ influx, and this can be inhibited by actinomycin D. Using yeast two hybrid system, two PKC receptor interacting proteins have been identified: p21ras farnesyltransferase (Wang et al., 1996), and immunophilin FKBP12 (Wang and Donahoe, 1994). Recent work has demonstrated that members of the Smad family are key components in mediating TGF-β downstream events. On TGF-β binding, Smads become phosphorylated, form hetero-oligomers, and translocate to the nucleus, where they affect the transcription of the target genes (for reviews, see Heldin et al., 1997; Massague et al., 1997). Early signaling events of TGF-β have also been shown to include the expression of immediate early genes, such as fos (Kerr et al., 1990), jun (Li et al., 1990), and myc (Pieterpol et al., 1990), at the transcriptional level. It is possible that certain aspects of TGF-β action may be secondary to this event.

Although many studies have characterized the effect of TGF-β on various cells, little is known concerning the molecular mechanisms responsible for TGF-β action. In this study, we investigated the mechanism by which TGF-β inhibits prolactin gene expression in GH3 cells. We present evidence supporting that inactivation of PKC activity as a major signaling event mediates TGF-β action.

**Experimental Procedures**

**Materials.** Recombinant human TGF-β2 was a generous gift from Dr. Robert C. Chang (Celtrix Pharmaceuticals, Santa Clara, CA). DNA probe for prolactin (pml-sp65 1) was kindly supplied by Dr. Richard A. Maurer (Oregon Health Sciences University, Portland, OR). The GH3 clonal rat pituitary cell line was purchased from American Type Culture Collection (Rockville, MD). Ham’s F-10 medium, fetal bovine serum, and horse serum were from Gibco (Grand Island, NY). TRI Reagent was from Molecular Research Center (Cincinnati, OH). Specific antibodies against PKC subtypes were from Transduction Laboratories (Lexington, KY). Histone H III-S and MBP were from Sigma Chemical (St. Louis, MO). P81 paper and DEAE-cellulose were from Whatman (Maidstone, England).

**Cell culture.** GH3 cells were cultured in Ham’s F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum. Monolayer cultures were maintained under air/CO2 in a humidified chamber at 37° using Falcon Primaria dishes or culture flasks pre-treated with 0.15% (w/v) Type II collagen.

**RNA preparation and Northern blotting.** Total RNA was prepared as follows. Approximately 5 × 10⁶ cells were lysed in 1 ml of TRI Reagent through repetitive pipetting. The homogenate was extracted with 0.2 ml of chloroform and centrifuged at 12,000 × g for 15 min at 4°. The aqueous phase containing the RNA was precipitated with isopropanol. The pellet was washed with 70% ethanol.

For Northern blot hybridization, RNA was denatured with formaldehyde/formaldehyde and applied at 20 μg/lane to a 1.2% agarose gel containing formaldehyde. After electrophoresis, RNA was blotted to a nitrocellulose filter and hybridized with 32P-labeled DNA. The radioactivity was detected by exposing the nitrocellulose paper to Kodak X-ray film. Alternatively, radioactivity associated with the band was analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Preparation of cytosolic and membrane PKC.** Preparation of cell fractions for PKC assay was performed as described previously (Fearon and Tashjian, 1985). GH3 cells were harvested and resuspended in 20 mM HEPES, pH 7.2, containing 118 mM NaCl, 4.6 mM KCl, 0.1 mg/ml leupeptin, 10 mM glucose, and 1 μM CaCl2 at a density of 0.5–2 × 10⁶ cells/ml. Cells were broken by passage through a 25-gauge needle eight times; the mixture was centrifuged at 12,000 × g for 8 min. The supernatant (cytosol) was separated by a DEAE-cellulose column equilibrated with 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.5 mM dithiothreitol (column buffer), and PKC was eluted with column buffer containing 75 mM NaCl. The particulate fraction was reususpended in the column buffer containing 1% Triton X-100, sonicated 5 see each time on ice for three times and incubated at 4° for 1 hr. Insoluble material was removed by centrifugation at 12,000 × g for 8 min, and the supernatant was separated by the DEAE-cellulose column as described above. PKC was eluted with column buffer containing 80 mM NaCl.

**PKC assay.** PKC activity was determined by measuring the incorporation of [32P]PO4 into histone H III-S or MBP as described previously (Fearon and Tashjian, 1985). Reaction was carried out in a final volume of 200 μl containing 5 μmol of Tris-HCl, pH 7.5, 1.5 μmol of magnesium acetate, 100 nmol of CaCl2, 25 μg of phosphatidylserine, 2 μg of 1,2-diacyl-sn-glycerol, 100 μg of histone H III-S or 40 μg of MBP, and ~6 × 10⁸ cpm of [γ-32P]ATP. The reaction was carried out at 30° for 15 min and terminated by adding 0.3 ml of 25% trichloroacetic acid. The mixture was filtered, and acid-insoluble material was collected on a Whatman P81 paper. The filter was air dried and washed stepwisely four times 1% phosphoric acid and once each with 10% and 5% trichloroacetic acid. Radioactivity retained on the filter was determined by a β-counter.

**Immunoprecipitation.** Cells were lysed in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, containing 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 20 mM NaF, 0.2 mM Na3VO4, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride) at 4° for 30 min. Cells were then broken by repeated aspiration through a 25-gauge needle, and the lysate was centrifuged at 12,000 × g for 30 min. To reduce the nonspecific interactions, the lysates were first incubated with protein A-Sepharose. After centrifugation, the supernatant (250 μg of protein) was mixed with 1 μg of subtype-specific PKC antibodies, and the reaction was carried out for 2 hr at 4° with end-to-end rotation. Protein A-Sepharose was then added, and the reaction was continued for another hour. The mixture was centrifuged, and the immunoprecipitate was reacted with 40 μl of kinase assay solution using MBP as substrate as described above at 37° for 30 min. The assay mixture was precipitated with trichloroacetic acid and filtered through a P81 paper.

**Western blotting.** After separation by SDS-polyacrylamide gel electrophoresis in a 10% gel, the proteins were transferred to a nitrocellulose paper. The paper was soaked in 3% nonfat milk at 4°
Results

Dose- and time dependent inhibition of prolactin mRNA expression by TGF-β2. It has been shown by Delidow et al. (1991) that TGF-β suppressed both basal and Ca²⁺-stimulated prolactin mRNA levels in GH₃ cells cultured in serum-free medium. We showed that in the presence of serum, TGF-β₂ dose-dependently inhibited prolactin mRNA accumulation after a 24-hr incubation with GH₃ cells (Fig. 1). Maximal inhibition was attained at a TGF-β₂ concentration of 5 ng/ml. Under such conditions, mRNA levels of GAPDH and GH, also found to be expressed in GH₃ cells, remained essentially unchanged. This suggests that inhibition of prolactin gene expression was a specific effect of TGF-β₂.

Time course studies showed that TGF-β₂ exerted its maximal effect at 12–24 hr after its addition (Fig. 2). We examined whether the continuous presence of the hormone during this period was necessary for prolactin mRNA repression. GH₃ cells were exposed to TGF-β₂ for various time periods and then switched to medium without TGF-β₂. Prolactin mRNA expression was analyzed at 24 hr after TGF-β₂ treatment. Results in Fig. 3 demonstrated that approximately the same extent of inhibition was attained in cells transiently exposed to TGF-β₂ for as short a period as 20 min (lane 2) as with its continuous presence (lane 8), suggesting that the conveyance of hormonal message from outside the cells to intracellular compartments was completed at an early time point.

Requirement of protein synthesis and extracellular Ca²⁺ for TGF-β action. The delayed response of prolactin mRNA expression resulting from the addition of TGF-β suggests the possible involvement of protein synthesis in the process. To test this possibility, GH₃ cells were preincubated with the protein synthesis inhibitor cycloheximide (10 μg/ml) before the addition of TGF-β. We have found that GH₃ cells lose their viability in the presence of cycloheximide for 24 hr. Because the continuous presence of TGF-β₂ is not necessary for prolactin mRNA inhibition, the experiment was performed as follows. Cells were incubated with 10 μg/ml cycloheximide for 30 min before the addition of TGF-β₂. One hour after TGF-β₂ treatment, the cells were switched to medium without cycloheximide and TGF-β₂, and prolactin mRNA levels were analyzed at 24 hr after TGF-β₂ addition. As shown in Fig. 4, although cycloheximide inhibited GAPDH mRNA expression, changes in prolactin mRNA levels in response to TGF-β₂ was completely abolished by cycloheximide (ratio of prolactin to GAPDH in the presence of TGF-β₂ is 96% of that in its absence). This result suggests that protein synthesis is required for the inhibition of prolactin mRNA accumulation by TGF-β₂.

We next determined the involvement of Ca²⁺ in TGF-β₂ signaling. The Ca²⁺ chelator EGTA was used to remove extracellular Ca²⁺. Because prolonged exposure to EGTA resulted in cell death, conditions similar to those used for the cycloheximide experiment were followed. Cells were treated with TGF-β₂ in the presence of EGTA for 1 hr and then switched to medium without TGF-β₂ and EGTA, and the prolactin mRNA expression was analyzed at 24 hr. As can be seen (Fig. 4), prolactin mRNA response to TGF-β could be blocked by EGTA (ratio of prolactin to GAPDH in the presence of TGF-β₂ is 87% of that in its absence). Previous studies indicate a role of L-type Ca²⁺ channels in prolactin gene regulation (Hinkle et al., 1988). In the presence of L-type

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**Fig. 1.** Dose-dependent inhibition of prolactin mRNA expression by TGF-β₂. GH₃ cells were treated with increasing concentrations of TGF-β₂ as indicated for 24 hr. Total cellular RNA was extracted, and 20 μg/lane was applied to an agarose gel. After electrophoresis, the RNA was blotted onto a nitrocellulose paper and hybridized with the ²⁵P-labeled DNA of prolactin (PRL). The same blot was reprobed for GH and GAPDH (left). The autoradiogram was scanned with a densitometer, and prolactin mRNA expression was normalized with that of GAPDH. Results are expressed as a percentage of control (at zero dose) and are plotted in log scale versus TGF-β₂ concentrations (right). Two other independent experiments gave similar results.
Ca\(^{2+}\) channel blockers nifedipine or verapamil, basal prolactin mRNA levels decreased substantially; however, the response to TGF-\(\beta_2\) persisted under such circumstances (data not shown). These results suggest that Ca\(^{2+}\) entry across the plasma membrane in TGF-\(\beta_2\)-elicited prolactin gene suppression may involve channels other than L-type Ca\(^{2+}\) channels.

**Involvement of PKC in the inhibition of prolactin gene expression by TGF-\(\beta_2\).** Activation of PKC has been shown to stimulate prolactin gene expression. As is indicated in Fig. 5, we also demonstrated that PMA at a low dose of 10 ng/ml [Fig. 5, PMA(L)] stimulated prolactin mRNA accumulation, and this effect was inhibited by TGF-\(\beta_2\). When cells were pretreated with a high dose of PMA (2 \(\mu\)M for 24 hr) to deplete the cellular PKC, the TGF-\(\beta_2\) inhibition of prolactin mRNA expression was shown to be significantly reduced [Fig. 5, PMA(H)]. We demonstrated that pretreatment of cells with PMA (2 \(\mu\)M, 24 hr) was able to abolish the ability of PMA in stimulating c-fos mRNA expression in GH\(_3\) cells (Fig. 6), suggesting that PMA-sensitive pathways were indeed inhibited under such treatment. Western blot analysis was used to examine whether there was subtype-specific PKC down-regulation after prolonged PMA treatment. As shown in Fig. 7, levels of several PMA-sensitive PKCs, including the conventional types (cPKC), PKCa and PKCy (Fig. 7A), and the novel types (nPKC), PKC\(\delta\) and PKCe (Fig. 7B), were substantially decreased in the PMA-treated cells. Interestingly, down-regulation of PKC\(\beta\), a conventional PKC subtype, was less evident (Fig. 7A), as was the novel-type PKC\(\mu\) (Fig. 7B). We also found that GH\(_3\) cells expressed atypical PKC (aPKC) isozymes \(\omega\) and \(\lambda\), and their expression was not affected by persistent PMA stimulation (Fig. 7C). Together, these results suggest that selective isoforms of PMA-sensitive PKCs were indeed down-regulated by the prolonged PMA treatment. These observations support the involvement of PKC isozymes in mediation of TGF-\(\beta_2\) signaling.

**Inhibition of PKC activity by TGF-\(\beta_2\).** The above results, showing that stimulation of PKC increased prolactin gene expression, coupled with the observation that PKC down-regulation blocked prolactin mRNA response to TGF-\(\beta_2\), prompted us to examine whether TGF-\(\beta_2\) inhibited prolactin gene expression by negatively regulating PKC activity. GH\(_3\) cells were treated with 5 ng/ml TGF-\(\beta_2\), and at various time periods, the cytosolic and particulate fractions were prepared. The activities of PKC in these fractions were measured using histone HIII-S as substrate after partially purified from the DEAE-cellulose column as described previously (Fearon and Tashjian, 1985). As indicated in Fig. 8A, both the cytosolic and membranous PKC activities were significantly decreased in the TGF-\(\beta_2\)-treated cells. Inhibition was found at 20 min after the addition of the hormone and continued to 24 hr, the last time point analyzed. The basal PKC levels during the 24-hr period remained essentially the same (data not shown). TGF-\(\beta_2\) did not affect the subcellular distribution of PKC because activity ratios of membrane to cytosolic PKC were not significantly changed throughout the 24-hr period of TGF-\(\beta_2\) treatment (Fig. 8A). Consistent with the finding that the sustained presence of TGF-\(\beta_2\) was not necessary for prolactin mRNA inhibition, the transient presence of TGF-\(\beta_2\) was demonstrated to down-regulate PKC activity. As shown in Fig. 8B, treatment of cells with TGF-\(\beta_2\) for 20 min resulted in a similar extent of inhibition on PKC activity as with its continuous presence.

An *in vitro* kinase assay of the immunoprecipitated PKCs was performed next to verify that activities of individual PKCs were indeed suppressed. Representative PKC isozymes were precipitated with subtype-specific antibodies, and the immunoprecipitates were assayed for kinase activities. It has been reported that histone HIII-S was not a particularly good substrate for some of the novel PKC isoforms, whereas MBP was a very good substrate for all PKC family members (Toker et al., 1994). MBP therefore was used as the substrate for the immunoprecipitated PKCs. Results in Table 1 indicate that TGF-\(\beta_2\) decreased the activities of several PKCs, with a greater extent of inhibition on the isozymes \(\alpha\) and \(\epsilon\). The
activities of PKCζ and PKCλ also were inhibited but to a lesser extent. On the other hand, no statistically significant inhibition was found on PKCδ (p > 0.2). A negative control performed on PKCδ was included. The antibody for PKCδ was unable to precipitate the enzyme; as a result, no detectable kinase activity could be obtained in the control and TGF-β2-treated cells.

To investigate mechanisms underlying PKC inhibition, we

![Fig. 3](image_url)

**Fig. 3.** Effect of TGF-β2 pretreatment times on the inhibition of prolactin mRNA expression. GH3 cells were incubated with 5 ng/ml of TGF-β2 for various times. Cells were washed and cultured in the absence of TGF-β2 for a total of 24 hr (including the preincubation time), and prolactin mRNA levels were determined by Northern blotting. Lanes 1–8, samples from TGF-β2 pretreatment times of 0 hr, 20 min, 40 min, 1 hr, 3 hr, 6 hr, 12 hr, and 24 hr, respectively.

![Fig. 4](image_url)

**Fig. 4.** Protein synthesis and extracellular Ca²⁺ are required for the TGF-β2-inhibited prolactin mRNA expression. GH3 cells were incubated in the presence (+) or absence (−) of TGF-β2 and analyzed for prolactin mRNA expression. Effect of protein synthesis on TGF-β2 action was analyzed by preincubating cells with cycloheximide (CHX, 10 μg/ml) for 30 min before the addition of TGF-β2. At 1 hr after TGF-β2 treatment, the cells were switched to medium without cycloheximide and TGF-β2, and prolactin mRNA levels were analyzed at 24 hr after TGF-β2 administration. Alternatively, cells were treated simultaneously with TGF-β2 and EGTA for 1 hr. They were then cultured in medium with neither TGF-β2 nor EGTA, and the prolactin mRNA expression was analyzed at 24 hr after TGF-β2 addition. The blot was reprobed with GAPDH and 18S rRNA to ensure equal amounts of RNA were loaded.

![Fig. 5](image_url)

**Fig. 5.** Effect of PMA pretreatment on TGF-β2-inhibited prolactin mRNA expression. GH3 cells were treated with a low dose of PMA [20 ng/ml PMA(L)] in the presence (+) or absence (−) of TGF-β2 for 24 hr, and prolactin or RNA levels were analyzed by Northern blotting. Alternatively, GH3 cells were pretreated with 2 μg/ml PMA for 24 hr [PMA(H)] before the addition of TGF-β2, and prolactin mRNA expression was examined by Northern blot analysis. Expression of 18S rRNA indicates equal amounts of RNA were loaded onto each lane.

![Fig. 6](image_url)

**Fig. 6.** Effect of prolonged PMA treatment on the PMA-stimulated c-fos mRNA induction. GH3 cells were pretreated with 2 μg/ml PMA for 24 hr to deplete PKC [PMA(H)], after which they were restimulated with 20 ng/ml PMA [PMA(L)] for 1 hr, and RNA was extracted and analyzed for c-fos expression by Northern blot analysis.

![Fig. 7](image_url)

**Fig. 7.** Effect of prolonged PMA pretreatment on PKC isozyme levels. GH3 cells were treated with (+) and without (−) PMA (2 μg/ml) for 24 hr. Total cell lysates were prepared as described in the text. The lysates (100 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis (10% gel), transferred to a nitrocellulose paper, and reacted with subtype-specific antibodies to PKCs as indicated. The blot was incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody, followed by reaction with enhanced chemiluminescence reagents and exposure to an X-ray film. Molecular masses of prestained marker proteins are shown in kDa, and the positions of PKC isozymes are indicated (arrows). A, Conventional PKCs. B, Novel PKCs. C, Atypical PKCs.
examined whether TGF-β2-induced changes in the protein levels of PKCe, the most abundant PKC isoenzyme present in GH3 cells (Akita et al., 1994). Cells were treated with and without TGF-β2 for 3 hr, and total cell extracts were immu-

noprecipitated with antibodies against PKCe. In vitro kinase assays were performed on the immunoprecipitate by reaction with [γ-32P]ATP. As indicated in Fig. 9 (right), although no phosphorylation was detected in the precipitates using the nonimmune IgG (lanes 1 and 2), phosphorylation was found to be associated with the anti-PKCε immunoprecipitates (lanes 3 and 4). A band with an apparent molecular mass of ~95 kDa was found, which probably represents autophosphorylated PKCe. Decreased phosphorylation on this band was demonstrated in the TGF-β2-treated cells. Other than PKCe, two proteins with reduced phosphorylation after TGF-β2 treatment were seen reproducibly; one migrates with an apparent molecular mass of >112 kDa, which could represent a PKCε-associated protein, and the other has a molecular mass of ~50 kDa, which comigrates with immunoglobulins. When aliquots of the in vitro kinase assay mixtures that were used in autophosphorylation experiments were separated on an SDS-polyacrylamide gel for immunoblot analysis of PKCe expression, the 95-kDa PKCe was specifically decorated. However, no change in the PKCe protein level could be detected in the presence or absence of TGF-β2 (Fig. 9, left). These results indicate that inhibition of PKCe activity by TGF-β2 may be mediated through post-translational regulation of the enzyme.

**Discussion**

Despite the findings that TGF-β plays fundamental roles in regulating many cellular activities, the understanding of the intracellular signaling events responsible for TGF-β action is much less comprehensive. In this study, we provide evidence supporting that extracellular Ca2+, PKC, and protein synthesis are involved in mediating TGF-β2-inhibited prolactin mRNA expression.

Results from the current study support the hypothesis that PKC may play a role in the inhibition of the prolactin gene expression by TGF-β2. First, PKC is a positive regulator in prolactin gene expression. Our data indicate that PMA stimulates prolactin mRNA levels, and this effect is inhibited by TGF-β2 (Fig. 5). Second, under the conditions of prolonged PMA treatment, where several PKC isozymes are shown to be down-regulated (Fig. 7) and functional PMA-sensitive PKC was found to be absent (Fig. 6), the TGF-β2-inhibited prolactin gene expression is significantly suppressed. Third, treatment of cells with TGF-β2 potently inhibits PKC activity. We have shown that activities of both the membrane and cytosolic PKC are significantly reduced whether cells are treated with TGF-β2 transiently or persistently; this is supported further by kinase assays of the immunoprecipitated PKC isozymes.

An analysis of PKC activity indicates that a substantial fraction of PKC is located in the membrane. This is consistent with the subtype-specific expression and distribution of PKC in GH pituitary tumor cells. It has been shown that PKCe is the most abundant isozyme in GH pituitary cells (Akita et al., 1994); its transcript level accounts for >70% of total PKC mRNA in these cells. Moreover, a significant portion of the enzyme is membrane associated in unstimulated GH cells. In addition to PKCe, we found that the activities of PKCα, PKCγ, and PKCζ also are suppressed, although to different degrees, by TGF-β2. The activity of PKCλ, however, does not seem to be affected by TGF-β2. These results indi-

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TABLE 1

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* Significant inhibition by TGF-β2.  
* N.D., no significant [32P] incorporation was detected.
icate that TGF-β₂ selectively affects multiple PKC isoenzymes. This is the first report demonstrating that the cytokine can exert differential inactivation on specific PKC isoenzymes. Currently, we are unable to pinpoint which of these PKC isoenzymes is responsible for the suppression by TGF-β₂ of prolactin gene expression. Because down-regulation of the atypical PKCs is not observed under prolonged PMA treatment, when essentially all of the cellular response to TGF-β₂ was blocked, then the atypical PKCs probably are not involved in mediation of the TGF-β₂ effect.

A number of studies have indirectly linked PKC to TGF-β signaling. The stimulation of hyaluronan biosynthesis in human fibroblast by TGF-β seems to require PKC activation (Suzuki et al., 1995). TGF-β activates PKC activity in a human colon carcinoma cell line (Chakrabarty, 1992). Alternatively, TGF-β has been shown to exert a negative modulation on PKC or the PKC-dependent pathway. For example, the proliferation of B cells stimulated by PMA can be inhibited by TGF-β; similarly, the TGF-β-stimulated phagocytosis of human retinal pigment epithelium is counteracted by the simultaneous presence of PMA. Nishikawa et al. (1993) have shown that TGF-β suppressed the PMA-stimulated phosphorylation of three endogenous proteins in primary epidermal cells. It was further demonstrated, based on substrate specificities, that the activities of nPKC, but not those of cPKC, are decreased in the presence of TGF-β (Nishikawa et al., 1993). These studies, which are based mostly on the inhibitor studies and PKC depletion experiments, suggest that PKCs may mediate multiple cellular responses to TGF-β. On the other hand, studies also indicate that some effects of TGF-β do not require PKC. For example, Ohitsuke and Massague (1992) have shown that the activation of plasminogen activator inhibitor-1 by TGF-β₁ is not affected by PKC depletion. Similarly, PKC is not necessary for the TGF-β-induced growth arrest in leukemia cell lines (Manzel and Macfarlane, 1997). However, in these studies, changes in PKC activity after TGF-β treatment were not determined; thus, it remains possible that PKC may still be targeted by TGF-β and mediate other effects of the hormone in these cells. Up to this date, information concerning the involvement of PKC in TGF-β signaling is limited, and the generality of PKC in mediating TGF-β signaling remains to be elucidated. Although as mentioned, some studies also claim that TGF-β can either activate or inhibit the activities of PKCs, sources of PKC used for activity determination in these studies are not pure; further work is required to permit a definitive conclusion on whether the PKC activities are indeed affected by TGF-β. By assaying the individually immunoprecipitated PKC subtypes, we provided direct evidence that the activities of several PKCs are significantly inhibited by TGF-β₂.

Very little is known regarding the mechanism underlying TGF-β₂ inhibition on PKC activity. TGF-β₂ does not seem to alter the subcellular distribution of PKC (Fig. 8). Subsequent stimulation with PMA at various times after TGF-β₂ treatment revealed that TGF-β₂ does not interfere with the PMA-induced translocation of the PKC (data not shown). Using PKC as a model for investigation, we have shown by immunoblotting that TGF-β₂ does not affect the level of the enzyme. On the other hand, autophosphorylation of the immunoprecipitated enzyme is significantly reduced after TGF-β₂ treatment. It seems that post-transcriptional mechanisms are involved in regulating PKC activity by TGF-β₂.

Several lines of evidence support a role of PKC in mediating the hormonal actions in normal and neoplastic pituitary cells. Estrogen, which stimulates pituitary cell growth and prolactin synthesis, has been shown to increase the calcium-dependent and -independent PKC mRNA expression in normal pituitary cells (Maeda and Lloyd, 1993). The suppression of prolactin mRNA expression by dopamine can be inhibited by prolonged TPA treatment, suggesting the involvement of PKC in dopamine signaling (Chuang et al., 1993). The signaling pathway of TRH, another physiological regulator of pituitary function, also involves PKC. It has been shown that overexpression of PKCe increases the TRH-stimulated secretion of prolactin in GH4C1 cells; moreover, TRH induces translocation of several PKC isoenzymes, including PKCa, PKCe, PKCc, and PKCd, from cytosol to membrane (Akita et al., 1994). Taken together, PKC may be involved in regulating not only the secretion and synthesis of prolactin but also lactotroph cell proliferation. Our finding that TGF-β inhibits the activity of multiple PKC isoenzymes, coupled with the finding of the presence of both TGF-β and its receptors in lactotropes, suggests that this hormone is potentially capable of interacting with many other physiological regulators in maintaining the normal function of lactotropes.

Other than PKC inhibition, our results support that extracellular Ca²⁺ and a newly synthesized protein are important in the action of TGF-β₂. TGF-β₂-inhibited prolactin mRNA could be blocked by EGTA; however, we are unable to detect

Fig. 9. Effect of TGF-β₂ on the protein and autophosphorylation activity of PKCe. GH₃ cells were treated with (+) or without (−) TGF-β₂ for 3 hr. At the end of the incubation, cells were lysed, and total cell extract was prepared as described in the text. The lysates were reacted with antibodies against PKCe or 2 hr and precipitated by the addition of protein A-Sepharose. The immunoprecipitates were incubated with PKC assay mixture in the presence of ³²P-ATP as described in the text, except that histone H III-S was not present. At the end of the reaction, equal amounts of the reaction mixture were applied separately onto two SDS-polyacrylamide gels (10%). Left, immunoblot analysis of PKCe levels. Molecular masses of prestained marker proteins are shown in kDa, and position of PKCe is indicated (arrow). Right, analysis of autophosphorylation levels by autoradiography. Lanes 1 and 2, nonimmune IgG precipitates. Lanes 3 and 4, PKCe precipitates.
any immediate changes in intracellular Ca\(^{2+}\) levels after TGF-\(\beta_2\) treatment. Data from this laboratory also show that cobalt, but not nifedipine or verapamil, blocks the TGF-\(\beta_2\) action. Similar observations have been demonstrated in the TGF-\(\beta\)-induced calcium influx in Rat-1 fibroblast cells (Muldoon et al., 1988). It has been shown that stimulation of Ca\(^{2+}\) influx by TGF-\(\beta\) is observed at incubation times of \(>1\) hr and is not mediated through L-type Ca\(^{2+}\) channels. Moreover, TGF-\(\beta\)-exerted Ca\(^{2+}\) influx is blocked by actinomycin D, suggesting that a newly transcribed gene product is required for the observed calcium response in Rat-1 cells. By analogy to the findings in Rat-1 cells, the putative newly synthesized protein may be required for the induction by TGF-\(\beta_2\) of Ca\(^{2+}\) influx in GH\(_3\) cells. Despite the fact that TGF-\(\beta\) could alter the expression of c-fos (Kerr et al., 1991), jun (Li et al., 1990), and myc (Pietenpol et al., 1990) at a very early stage, we are unable to detect any changes in mRNA expression of these genes in the TGF-\(\beta_2\)-treated GH\(_3\) cells (data not shown). Therefore, the synthesis of proteins other than these oncogenes may be involved in the TGF-\(\beta_2\) action. Although the exact coordination among PKC, protein synthesis, and Ca\(^{2+}\) influx that eventually led to prolactin mRNA repression remains to be clarified, we have nevertheless provided substantial evidence that PKC is an early signaling mediator of prolactin mRNA inhibition by TGF-\(\beta_2\) in GH\(_3\) cells.

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