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Evidence for the Involvement of Protein Kinase C in the Inhibition of Prolactin Gene Expression by Transforming Growth Factor-β_2

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)-β_2 inhibited prolactin mRNA expression in GH_3 rat pituitary tumor cells. Maximal inhibition was observed with cells exposed to 5 ng/ml TGF-β_2 for 24 hr. Continuous presence of the hormone during the entire period was not necessary because exposure of cells to TGF-β_2 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-β_2 could be abolished by cycloheximide or EGTA, suggesting the requirement of a newly synthesized protein and extracellular Ca^{2+}. The response of prolactin mRNA to TGF-β_2 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-β_2 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-β_2. Inhibition of PKC did not require the sustained presence of TGF-β_2. *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-β_2-treated cells, whereas that of PKCλ was not affected. Western blotting did not reveal any change in PKCϵ steady state protein levels, suggesting TGF-β_2 inhibits PKC activity through a post-translational mechanism. Our results support that inhibition of PKC activity is an early event mediating TGF-β_2-inhibited prolactin mRNA expression in GH_3 cells.

Prolactin is a lactotropic polypeptide hormone originally found to be secreted by the pituitary. Recent studies indicate that thymocytes (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-β_1, TGF-β_2, and TGF-β_3, 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

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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 Ca^{2+}-stimulated prolactin mRNA levels through transcriptional regulation in GH_3 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 kinase family, was recently shown to be activated by TGF-β (Yamaguchi *et al*., 1995). In Mv1Lu 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 phosphatidylinositide turnover (Muldoon *et al*., 1988). 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 Ca^{2+} influx, and this can be inhibited by actinomycin D. Using yeast two hybrid system, two TGF-β receptor interacting proteins have been identified: $p21^{ras}$ 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 heterooligomers, 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* (Pietenpol *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_2 inhibits prolactin gene expression in GH_3 cells. We present evidence supporting that inactivation of PKC activity as a major signaling event mediating TGF-β_2 action.

Experimental Procedures

Materials. Recombinant human TGF-β_2 was a generous gift from Dr. Robert C. Chang (Celtrix Pharmaceuticals, Santa Clara, CA). DNA probe for prolactin (prl-sp65 1) was kindly supplied by Dr. Richard A. Maurer (Oregon Health Sciences University, Portland, OR). The GH_3 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. GH_3 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/CO_2 in a humidified chamber at 37° using Falcon Primaria dishes or culture flasks pretreated with 0.15% (w/v) Type II collagen.

RNA preparation and Northern blotting. Total RNA was prepared as follows. Approximately 5×10^6 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 formamide/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 ^{32}P-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). GH_3 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 $CaCl_2$ at a density of $0.5–2 \times 10^7$ 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 resuspended in the column buffer containing 1% Triton X-100, sonicated 5 sec 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 [^{32}P]PO_4 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 $CaCl_2$, 25 μg of phosphatidylserine, 2 μg of 1,2-dioctanoyl-Sn-glycerol, 100 μg of histone H III-S or 40 μg of MBP, and $\sim 6 \times 10^5$ cpm of [γ-^{32}P]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 Na_3VO_4, 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°

overnight to decrease nonspecific binding and then made to react with specific antibodies against PKC isozymes diluted in 3% nonfat milk for 45 min. The membrane was washed twice for 7 min with 10 mM Tris·HCl/150 mM NaCl, pH 8.0, containing 0.1% Tween 20 (TBST), followed by an incubation with horseradish peroxidase-conjugated goat anti-mouse antibody at 1:3000 dilution in 3% nonfat milk for 30 min. The membrane was washed twice for 15 min with TBST and twice for 5 min with TBST. Signals were detected by treating with ECL reagents (Amersham International, Buckinghamshire, UK) for 1 min, followed by exposure to an X-ray film.

Results

Dose- and time dependent inhibition of prolactin mRNA expression by TGF-β_2. It has been shown by Delidow *et al*. (1991) that TGF-β suppressed both basal and Ca^{2+}-stimulated prolactin mRNA levels in GH_3 cells cultured in serum-free medium. We showed that in the presence of serum, TGF-β_2 dose-dependently inhibited prolactin mRNA accumulation after a 24-hr incubation with GH_3 cells (Fig. 1). Maximal inhibition was attained at a TGF-β_2 concentration of 5 ng/ml. Under such conditions, mRNA levels of GAPDH and GH, also found to be expressed in GH_3 cells, remained essentially unchanged. This suggests that inhibition of prolactin gene expression was a specific effect of TGF-β_2.

Time course studies showed that TGF-β_2 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. GH_3 cells were exposed to TGF-β_2 for various time periods and then switched to medium without TGF-β_2. Prolactin mRNA expression was analyzed at 24 hr after TGF-β_2 treatment. Results in Fig. 3 demonstrated that approximately the same extent of inhibition was attained in cells transiently exposed to TGF-β_2 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 Ca^{2+} 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, GH_3 cells were preincubated with the protein synthesis inhibitor cycloheximide (10 μg/ml) before the addition of TGF-β. We have found that GH_3 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-β_2. One hour after TGF-β_2 treatment, the cells were switched to medium without cycloheximide and TGF-β_2, and prolactin mRNA levels were analyzed at 24 hr after TGF-β_2 addition. As shown in Fig. 4, although cycloheximide inhibited GAPDH mRNA expression, changes in prolactin mRNA levels in response to TGF-β_2 was completely abolished by cycloheximide (ratio of prolactin to GAPDH in the presence of TGF-β_2 is 96% of that in its absence). This result suggests that protein synthesis is required for the inhibition of prolactin mRNA accumulation by TGF-β_2.

We next determined the involvement of Ca^{2+} in TGF-β_2 signaling. The Ca^{2+} chelator EGTA was used to remove extracellular Ca^{2+}. 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-β_2 in the presence of EGTA for 1 hr and then switched to medium without TGF-β_2 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-β_2 is 87% of that in its absence). Previous studies indicate a role of L-type Ca^{2+} channels in prolactin gene regulation (Hinkle *et al*., 1988). In the presence of L-type

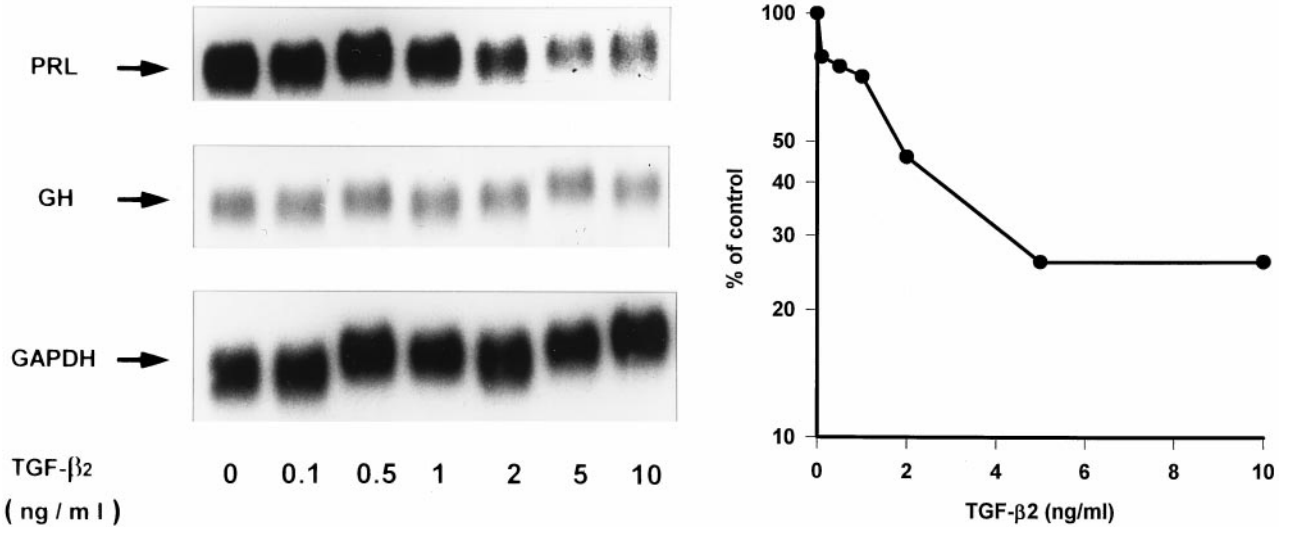

Fig. 1. Dose-dependent inhibition of prolactin mRNA expression by TGF-β_2. GH_3 cells were treated with increasing concentrations of TGF-β_2 as indicated for 24 hr. Total cellular RNA was extracted, and 20 μg/lane was applied to an agarose gel. After electrophoresis, the RNA was blotted onto a nitrocellulose paper and hybridized with the ^{32}P-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-β_2 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-β_2 persisted under such circumstances (data not shown). These results suggest that Ca^{2+} entry across the plasma membrane in TGF-β_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-β_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-β_2. When cells were pretreated with a high dose of PMA (2 μM for 24 hr) to deplete the cellular PKC, the TGF-β_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 μM, 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), PKCα and PKCγ (Fig. 7A), and the novel types (nPKC), PKCδ and PKCϵ (Fig. 7B), were substantially decreased in the PMA-treated cells. Interestingly, down-regulation of PKCβ, a conventional PKC subtype, was less evident (Fig. 7A), as was the novel-type PKCμ (Fig. 7B). We also found that GH_3 cells expressed atypical PKC (aPKC) isozymes ω and λ, 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-β_2 signaling.

Inhibition of PKC activity by TGF-β_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-β_2, prompted us to examine whether TGF-β_2 inhibited prolactin gene expression by negatively regulating PKC activity. GH_3 cells were treated with 5 ng/ml TGF-β_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-β_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-β_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-β_2 treatment (Fig. 8A). Consistent with the finding that the sustained presence of TGF-β_2 was not necessary for prolactin mRNA inhibition, the transient presence of TGF-β_2 was demonstrated to down-regulate PKC activity. As shown in Fig. 8B, treatment of cells with TGF-β_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-β_2 decreased the activities of several PKCs, with a greater extent of inhibition on the isozymes α and ϵ. The

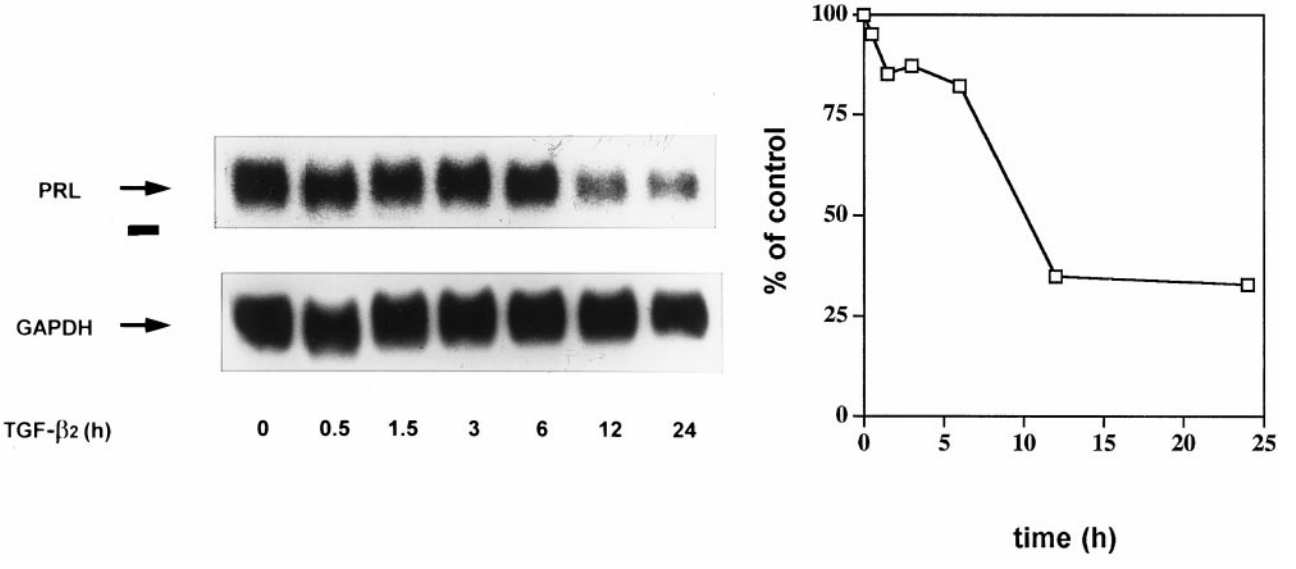

Fig. 2. Time course of prolactin mRNA inhibition by TGF-β_2. GH_3 cells were treated with TGF-β_2 (5 ng/ml) for up to 24 hr. At the indicated time intervals, total RNA was extracted and analyzed for prolactin mRNA expression as described in the legend to Fig. 1. The same blot was reprobed for GAPDH to ensure equal amounts of RNA were loaded in all samples (*left*). The autoradiogram was scanned with a densitometer, and prolactin mRNA expression was normalized with that of GAPDH. Results are expressed as percentage of control (at zero time) and are plotted against the time of TGF-β_2 treatment (*right*). Two other independent experiments gave similar results.

activities of PKCζ and PKCμ also were inhibited but to a lesser extent. On the other hand, no statistically significant inhibition was found on PKCλ ($p > 0.2$). A negative control

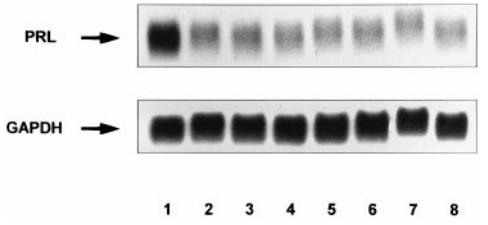

Fig. 3. Effect of TGF-β_2 pretreatment times on the inhibition of prolactin mRNA expression. GH_3 cells were incubated with 5 ng/ml of TGF-β_2 for various times. Cells were washed and cultured in the absence of TGF-β_2 for a total of 24 hr (including the preincubation time), and prolactin mRNA levels were determined by Northern blotting. *Lanes 1–8*, samples from TGF-β_2 pretreatment times of 0 hr, 20 min, 40 min, 1 hr, 3 hr, 6 hr, 12 hr, and 24 hr, respectively.

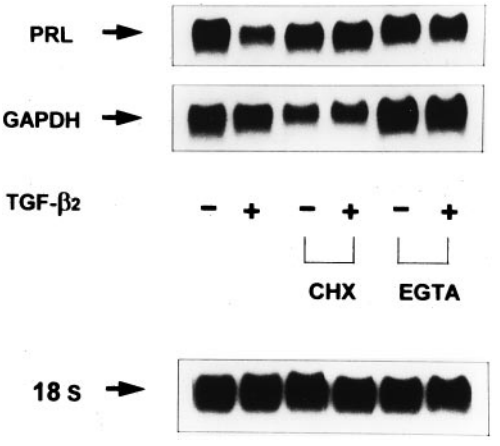

Fig. 4. Protein synthesis and extracellular Ca^{2+} are required for the TGF-β_2-inhibited prolactin mRNA expression. GH_3 cells were incubated in the presence (+) or absence (−) of TGF-β_2 and analyzed for prolactin mRNA expression. Effect of protein synthesis on TGF-β_2 action was analyzed by preincubating cells with cycloheximide (*CHX*, 10 μg/ml) for 30 min before the addition of TGF-β_2. At 1 hr after TGF-β_2 treatment, the cells were switched to medium without cycloheximide and TGF-β_2, and prolactin mRNA levels were analyzed at 24 hr after TGF-β_2 administration. Alternatively, cells were treated simultaneously with TGF-β_2 and EGTA for 1 hr. They were then cultured in medium with neither TGF-β_2 nor EGTA, and the prolactin mRNA expression was analyzed at 24 hr after TGF-β_2 addition. The blot was reprobed with GAPDH and 18S rRNA to ensure equal amounts of RNA were loaded.

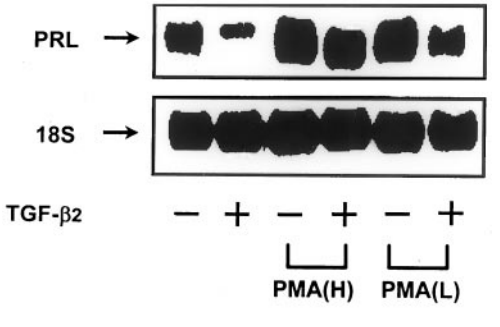

Fig. 5. Effect of PMA pretreatment on TGF-β_2-inhibited prolactin mRNA expression. GH_3 cells were treated with a low dose of PMA [20 ng/ml PMA(L)] in the presence (+) or absence (−) of TGF-β2 for 24 hr, and prolactin or RNA levels were analyzed by Northern blotting. Alternatively, GH_3 cells were pretreated with 2 μg/ml PMA for 24 hr [*PMA(H)*] before the addition of TGF-β_2, and prolactin mRNA expression was examined by Northern blot analysis. Expression of 18S rRNA indicates equal amounts of RNA were loaded onto each lane.

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-β_2-treated cells.

To investigate mechanisms underlying PKC inhibition, we

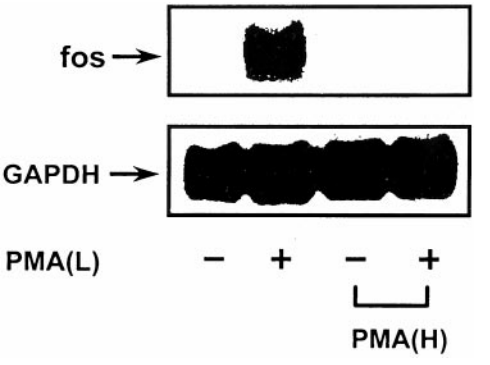

Fig. 6. Effect of prolonged PMA treatment on the PMA-stimulated c-*fos* mRNA induction. GH_3 cells were pretreated with 2 μg/ml PMA for 24 hr to deplete PKC [*PMA(H)*], after which they were restimulated with 20 ng/ml PMA [*PMA(L)*] for 1 hr, and RNA was extracted and analyzed for c-*fos* expression by Northern blot analysis.

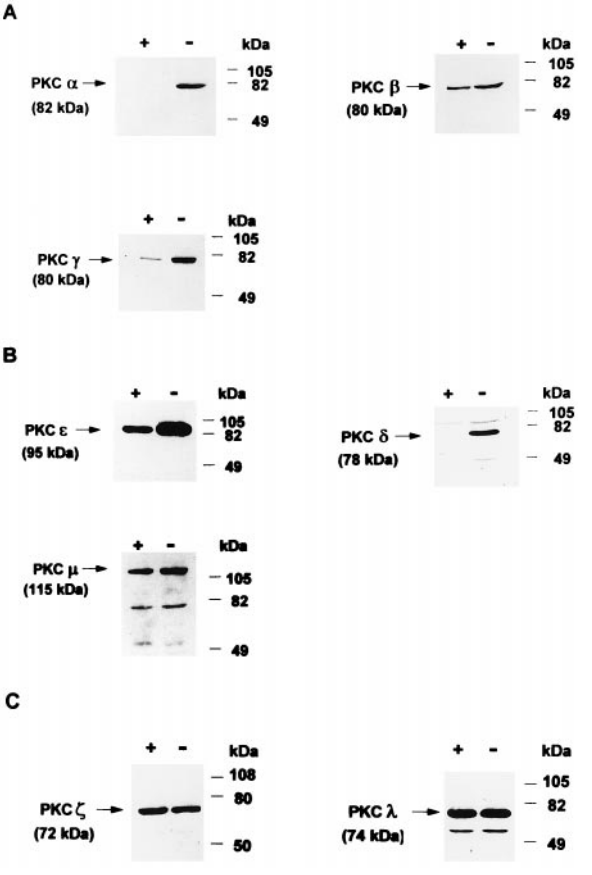

Fig. 7. Effect of prolonged PMA pretreatment on PKC isozyme levels. GH_3 cells were treated with (+) and without (−) PMA (2 μg/ml) for 24 hr. Total cell lysates were prepared as described in the text. The lysates (100 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis (10% gel), transferred 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-β_2 induced changes in the protein levels of PKCε, the most abundant PKC isoenzyme present in GH_3 cells (Akita *et al.*, 1994). Cells were treated with and without TGF-β_2 for 3 hr, and total cell extracts were immunoprecipitated with antibodies against PKCε. *In vitro* kinase assays were performed on the immunoprecipitate by reaction with [γ-^{32}P]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 PKCε. Decreased phosphorylation on this band was demonstrated in the TGF-β_2-treated cells. Other than PKCε, two proteins with reduced phosphorylation after TGF-β_2 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 PKCε expression, the 95-kDa PKCε was specifically decorated. However, no change in the PKCε protein level could be detected in the presence or absence of TGF-β_2 (Fig. 9, *left*). These results indicate that inhibition of PKCε activity by TGF-β_2 may be mediated through posttranslational regulation of the enzyme.

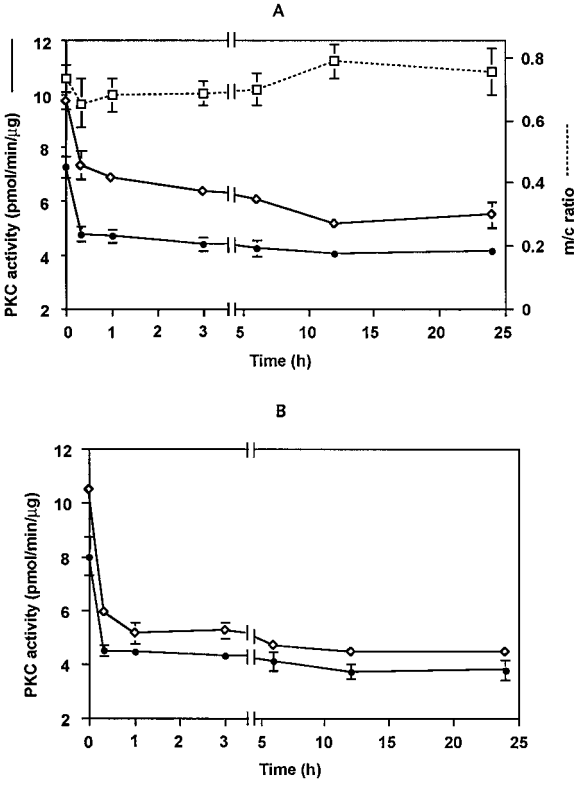

Fig. 8. Time-dependent inhibition of PKC activity by TGF-β_2. A, GH_3 cells were treated with TGF-β_2 (5 ng/ml) for various times as indicated. B, Alternatively, GH_3 cells were preincubated with 5 ng/ml TGF-β_2 for the indicated times, cells were washed and cultured in the absence of TGF-β_2 for a total of 24 hr (including the preincubation time). At the end of the incubation, cells were lysed, and the membrane (●) and the cytosolic (◇) fractions were prepared as described in the text. PKC was partially purified by the DEAE-cellulose column (Fearon and Tashjian, 1985). Kinase activity was assayed by measuring the incorporation of [^{32}P]PO_4 using histone H III-S as a substrate as described in the text. Ratios of membrane to cytosolic PKC activity (m/c ratio; □) were calculated based on the data obtained. Each point represents the mean ± standard error from four independent experiments, each with triplicate determinations.

TABLE 1

Effect of TGF-β2 on the activities of PKC isozymes

GH_3 cells were treated with or without TGF-β2 (5 ng/ml) for 3 hr. Cells were harvested, and total cell lysates were prepared. Individual PKC was precipitated with subtype-specific antibodies followed by reaction with protein A-coupled Sepharose 4B. The activity of PKC in the immunoprecipitate was determined by the incorporation of [γ-^{32}P]ATP to myelin basic protein as described in the text; it is expressed as pmol of ^{32}P incorporated/min/μg of immunoprecipitate. Data are represented as mean ± standard deviation of three independent experiments, each with triplicate determinations.

Isozyme	PKC activity		P, Student's *t* test
	Control	TGF-β2	
	pmol/min/μg		
α	16.3 ± 0.39	9.43 ± 0.41	<0.001[a]
ε	14.1 ± 0.27	8.63 ± 0.04	<0.001[a]
μ	2.90 ± 0.10	2.16 ± 0.08	<0.005[a]
ξ	2.20 ± 0.03	1.76 ± 0.05	<0.005[a]
λ	4.33 ± 0.46	3.86 ± 0.41	>0.2[b]
δ	N.D.	N.D.	

[a] Significant inhibition by TGF-β2.
[b] No significant inhibition by TGF-β2.
N.D., no significant ^{32}P incorporation was detected.

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 Ca^{2+}, PKC, and protein synthesis are involved in mediating TGF-β_2-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-β_2. 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-β_2 (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-β_2-inhibited prolactin gene expression is significantly suppressed. Third, treatment of cells with TGF-β_2 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-β_2 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 PKCε 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 PKCε, we found that the activities of PKCα, PKCμ, and PKCζ also are suppressed, although to different degrees, by TGF-β_2. The activity of PKCλ, however, does not seem to be affected by TGF-β_2. These results indi-

cate that TGF-β_2 selectively affects multiple PKC isozymes. This is the first report demonstrating that the cytokine can exert differential inactivation on specific PKC isozymes. Currently, we are unable to pinpoint which of these PKC isozymes is responsible for the suppression by TGF-β_2 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-β_2 was blocked, then the atypical PKCs probably are not involved in mediation of the TGF-β_2 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, Ohtsuke 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 mediates 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-β_2.

Very little is known regarding the mechanism underlying TGF-β_2 inhibition on PKC activity. TGF-β_2 does not seem to alter the subcellular distribution of PKC (Fig. 8). Subsequent stimulation with PMA at various times after TGF-β_2 treatment revealed that TGF-β_2 does not interfere with the PMA-induced translocation of the PKC (data not shown). Using PKCϵ as a model for investigation, we have shown by immunoblotting that TGF-β_2 does not affect the level of the enzyme. On the other hand, autophosphorylation of the immunoprecipitated enzyme is significantly reduced after TGF-β_2 treatment. It seems that post-transcriptional mechanisms are involved in regulating PKCϵ activity by TGF-β_2.

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 PKCϵ increases the TRH-stimulated secretion of prolactin in GH4C1 cells; moreover, TRH induces translocation of several PKC isoenzymes, including PKCα, PKCβ, PKCδ, and PKCϵ, 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 Ca^{2+} and a newly synthesized protein are important in the action of TGF-β_2. TGF-β_2-inhibited prolactin mRNA could be blocked by EGTA; however, we are unable to detect

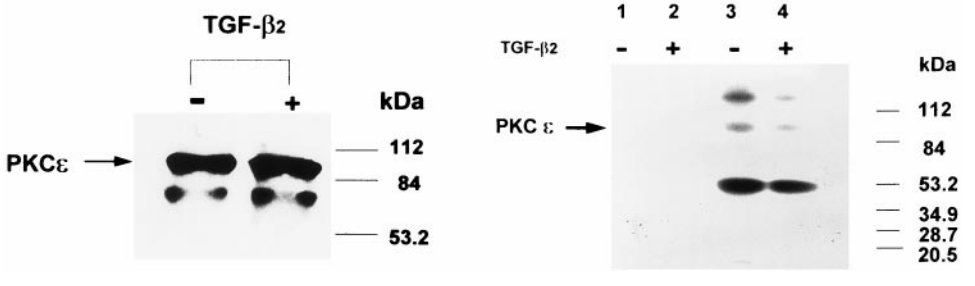

Fig. 9. Effect of TGF-β_2 on the protein and autophosphorylation activity of PKCϵ. GH_3 cells were treated with (+) or without (−) TGF-β_2 for 3 hr. At the end of the incubation, cells were lysed, and total cell extract was prepared as described in the text. The lysates were reacted with antibodies against PKCϵ for 2 hr and precipitated by the addition of protein A-Sepharose. The immunoprecipitates were incubated with PKC assay mixture in the presence of ^{32}P-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 PKCϵ levels. Molecular masses of prestained marker proteins are shown in kDa, and position of PKCϵ is indicated (*arrow*). *Right*, analysis of autophosphorylation levels by autoradiography. *Lanes 1 and 2*, nonimmune IgG precipitates. *Lanes 3 and 4*, PKCϵ precipitates.

any immediate changes in intracellular Ca^{2+} levels after TGF-β_2 treatment. Data from this laboratory also show that cobalt, but not nifedipine or verapamil, blocks the TGF-β_2 action. Similar observations have been demonstrated in the TGF-β-induced calcium influx in Rat-1 fibroblast cells (Muldoon *et al*., 1988). It has been shown that stimulation of Ca^{2+} influx by TGF-β is observed at incubation times of >1 hr and is not mediated through L-type Ca^{2+} channels. Moreover, TGF-β-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-β_2 of Ca^{2+} influx in GH_3 cells. Despite the fact that TGF-β 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-β_2-treated GH_3 cells (data not shown). Therefore, the synthesis of proteins other than these oncoproteins may be involved in the TGF-β_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-β_2 in GH_3 cells.

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Send reprint requests to: Dr. Fung-Fang Wang, Institute of Biochemistry, National Yang-Ming University, Shih-Pai, Taipei, Taiwan 11221. E-mail: ffwang@ym.edu.tw