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TGF-β Induced Hyaluronan Synthesis in Orbital Fibroblasts Involves Protein Kinase C βII Activation In Vitro

Hwai-Shi Wang,[1]* Wen-Hsiu Tung,[1] Kam-Tsun Tang,[2] Yi-Kuei Wong,[1] Ging-Jye Huang,[1] Jiahn-Chun Wu,[3] Yi-Jhih Guo,[1] and Chin-Chang Chen[1]

[1]Institute of Anatomy and Cell Biology, School of Medicine, Yang-Ming University, Taipei, Taiwan
[2]Department of Internal Medicine, Veterans General Hospital, Taipei, Taiwan
[3]Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei, Taiwan

Abstract Graves' ophthalmopathy is accompanied by hyaluronan (HA) accumulation in the orbital space and infiltration of immunocompetent cells and cytokines, including IFN-γ, IL-1β, and TGF-β. We examined the signal transduction pathways by which TGF-β induces HA synthesis in normal orbital fibroblasts, orbital fibroblasts from patients with Graves' ophthalmopathy, and abdominal fibroblasts. Calphostin C inhibited the stimulation of HA synthesis by TGF-β. Phorbol 12-myristate 13-acetate (PMA) activation of PKC stimulated HA production. The effects of TGF-β and PMA were not synergistic. Stimulation by TGF-β and PMA were dependent on protein synthesis and their effects were inhibited by cycloheximide. Since TGF-β-induced HA synthesis was inhibited by BAPTA or by PKC inhibitors, a calcium-dependent PKC was most likely involved. The PKA inhibitor H-89 enhanced TGF-β- and PMA-induced HA synthesis, thus showing that communication between the PKA and PKC pathways was evident. TGF-β stimulated the translocation of PKCβII to the cell membrane. PKCβII, a key enzyme in the regulation of HA synthesis by TGF-β, might be an appropriate target for therapeutic compounds to be used to treat Graves' ophthalmopathy accompanied by inflammation. J. Cell. Biochem. 95: 256–267, 2005.

Key words: orbital fibroblasts; TGF-β; hyaluronan; PKCβII; Graves' ophthalmopathy

Hyaluronan (HA), a linear polysaccharide composed of D-glucuronic acid and *N*-acetyl-D-glucosamine residues, is a characteristic component of the extracellular matrix [Laurent and Fraser, 1992]. HA plays an important part in wound healing [Okasala et al., 1995], embryonic development [Underhill et al., 1993], tumor growth [Bartolazzi et al., 1994], and synovial fluid viscosity [Swann et al., 1974]. HA also regulates cell–cell adhesion, cellular spatial orientation, migration, proliferation, and differentiation [Comper and Laurent, 1978; Love et al., 1979; Orkin et al., 1982; Brecht et al., 1986; McGuire et al., 1987; Laurent and Fraser, 1992]. HA is synthesized at the cell surface by membrane-bound enzyme, namely HA synthase (HAS), which is part of a multigene family that encodes various distinct isozymes, including HAS1 [Itano and Kimata, 1996; Shyjan et al., 1996], HAS2 [Spicer et al., 1996; Watanabe and Yamaguchi, 1996; Nishida et al., 1999], and HAS3 [Spicer et al., 1997].

Increased amounts of HA are present in the retroocular space of patients with Graves' ophthalmopathy, an autoimmune disease characterized by an accumulation of glycosaminoglycan (GAG; predominantly HA) in the retroocular connective tissue. GAG accumulation increases the volume of tissue in the orbit, leading to proptosis [Smith et al., 1989; Bahn

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*Correspondence to: Hwai-Shi Wang, PhD, Department of Anatomy, School of Medicine, Peitou, Taipei, Taiwan, R.O.C. E-mail: hswang@ym.edu.tw

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and Heufelder, 1992]. The retroocular space is infiltrated by lymphocytes, macrophages, and mast cells [Bahn and Heufelder, 1992]. Cytokines such as IFN-γ, IL-1, and TNF-α are present in orbital connective tissue of patients with ophthalmopathy [Heufelder and Bahn, 1994]. IL-1α, transforming growth factor β (TGF-β), and IFN-γ can up-regulate GAG synthesis in cultured human orbital fibroblasts [Campbell, 1984; Smith et al., 1991; Imai et al., 1992; Korducki et al., 1992]. These hydrophilic macromolecules increase the volume of orbital tissue and produce the clinical manifestations of Graves' ophthalmopathy [Bahn and Heufelder, 1993; Heufelder, 1993, 1995; Ajjan et al., 1997].

TGF-β, an important cytokine involved in cell growth and differentiation [Ignotz and Massague, 1985; Sporn and Roberts, 1985], is involved in tissue repair and fibrosis by regulating extracellular matrix production [Locci et al., 1999]. TGF-β has also been shown to contribute to the pathogenesis of autoimmune diseases such as systemic lupus erythematosus and glomerulosclerosis [Roberts, 1998]. The cellular actions of TGF-β on HA synthesis are diverse.

TGF-β stimulates HA synthesis in skin fibroblasts [Ellis et al., 1997], human ito cells [Ueki et al., 1998], liver parenchymal cells [Roth et al., 1998], and human malignant mesothelioma [Tzanakakis et al., 1997; Ellis and Schor, 1998; Roth et al., 1998; Ueki et al., 1998]. HA synthesis can be inhibited by TGF-β1 in human rheumatoid fibroblasts [Kawakami et al., 1998]. PKC [Klews and Prehm, 1994; Suzuki et al., 1995] or c-AMP dependent protein kinases [Honda et al., 1993; Imai et al., 1994; Klews and Prehm, 1994; Suzuki et al., 1995] may be involved in activating HA synthesis. TGF-β activates various serine/threonine kinases [Massague, 1996; Massague and Weis-Garcia, 1996] as well as a number of PKA pathways [Wang et al., 1998]. It has been found that TGF-β stimulates HA synthesis in human fibroblasts [Ellis et al., 1997]; however, the involved transduction pathways are not clear. Our previous study showed that phorbol 12-myristate 13-acetate (PMA), a PKC activator, stimulates HA synthesis in orbital fibroblasts [Wong et al., 2001]. Forskolin, a PKA activator, stimulates HA synthesis in rabbit pericardial mesothelial cells [Honda et al., 1993]. Although stimulation of HA by TGF-β is well recognized, the signaling pathways by which TGF-β regulates HA synthesis are poorly understood.

PKC, a family of serine/threonine kinases, plays a variety of crucial roles in various signal transduction pathways, including those regulating cell growth and differentiation. The PKC family is subdivided into three major classes: the conventional PKCs (cPKCs), the novel PKCs (nPKCs), and the atypical PKCs (aPKCs). All members of the family possess a highly conserved catalytic domain and a variable regulatory region. The regulatory region of the cPKCs contains a Ca^{2+}-binding domain that accounts for the Ca^{2+} sensitivity of these isoforms. The cPKCs comprise α, β, and γ isoforms, which show Ca^{2+}- and phospholipid-dependent enzyme activity and can be activated by phosphatidylserine (PS), diacylglycerol (DAG), or PMA. The nPKC isozymes are activated by PS, DAG, or PMA but not by Ca^{2+}. The aPKCs are not effected by Ca^{2+} and are unresponsive to DAG or PMA; these isoforms are dependent on PS for activation [Berry and Nishizuka, 1990; Hug and Sarre, 1993; Dekker and Parker, 1994; Newton, 1995].

Fibroblasts derived from different anatomical sites have different biological responses to cytokines, including changes in cell morphology [Smith et al., 1995a,b], HA synthesis [Smith et al., 1994], and the synthesis of specific proteins [Cao et al., 1998]. By using inhibitors, activators and Ca^{2+} chelators, we demonstrated that TGF-β-induced HA synthesis is mediated by PKCβII and Ca^{2+} in normal orbital fibroblasts, orbital fibroblasts from patients with Graves' ophthalmopathy and abdominal fibroblasts. The results also indicate that the levels of response to TGF-β were the same for orbital, Graves' and abdominal fibroblasts. Inhibition of protein synthesis with cycloheximide decreased the rate of HA synthesis. TGF-β-induced HA synthesis in fibroblasts was dependent on the presence of Ca^{2+} and the activation of PKCβII; thus cross-talk between PKA and PKC pathways was evident.

MATERIALS AND METHODS

Materials

Recombinant TGF-β was obtained from R&D (Minneapolis, MN). PMA, cycloheximide, and forskolin were purchased from Sigma (St. Louis, MO). The PKC inhibitor calphostin C was purchased from Calbiochem (San Diego, CA). MEM and other cell culture reagents were from Gibco (Gaithersburg, MD). DuPont-NEN Life

Science (Boston, MA) supplied the [^{3}H]glucosamine hydrochloride. The BCA protein assay kit was purchased from Pierce (Rockford, IL). The cDNA synthesis kits were purchased from Amersham Bioscience (Uppsala, Sweden). Rabbit anti-PKC antisera were obtained from Gibco and FITC-conjugated goat anti-rabbit IgG from Vector (Burlingame, CA).

Cell Culture

Fibroblast cultures were initiated from tissue explants obtained during the course of orbital surgery from patients with or without Graves' ophthalmopathy or from punch dermal biopsies provided by Dr. T.J. Smith (Albany Medical Center, NY). Institutional Review Board approval at both institutions was obtained for these activities. Cells were grown in MEM supplemented with 10% FBS, penicillin (100 U/ml)/streptomycin (100 μg/ml), nystatin (50 U/ml), and L-glutamine (2 mM) in a humidified incubator at 37°C with 5% CO_2. The medium was changed every 3–4 days and the cells were used before passage 12.

[^{3}H]GAG Assay

Human orbital fibroblasts were plated on sterile 60-mm diameter plastic dishes and allowed to reach confluence before being shifted to medium supplemented with 1% FBS with or without TGF-β or other test agents such as PMA, cycloheximide, or forskolin. When used, the PKC inhibitor calphostin C or the PKA inhibitor H-89 was added 1 h before other treatments.

To determine the effects of extracellular calcium, fibroblasts were plated in 5 ml of MEM supplemented with 1% FBS. Calcium concentrations, ranging from 0.05 to 4 mM, were adjusted by the addition of $CaCl_2$ to the Ca^{2+}-free MEM pre-treated with Chelex-100 (Bio-Rad, Richmond, CA) for 16 h. After treatment, the cultures were labeled with [^{3}H]glucosamine (1 μCi/ml) for 16 h. The medium was removed at the time of harvest and the cell layer was rinsed with PBS. The removed medium and PBS were then pooled. The cell layer was removed from the substratum using 2 ml of 0.2N NaOH and a cell scraper and the cells were disrupted by sonication.

Quantification of radiolabeled GAG was performed as described previously [Smith et al., 1989]. Briefly, an aliquot of the solubilized cell layer was taken for cell protein quantification. The medium and the remainder of the solubilized cell layer were combined and subjected to pronase digestion (1 mg/ml) in 100 mM Tris buffer (pH 8.0) at 50°C overnight. The samples were cooled to 4°C and TCA was added to a final concentration of 5% (w/v). After incubation for 1 h on ice, the samples were centrifuged, and the supernatant of acid-soluble material was dialyzed against cold water for 2 days. The amount of radiolabeled GAG in the retained samples, which is defined as total GAG, was quantified by liquid scintillation [Smith et al., 1989].

RNA Extraction and RT-PCR

Human orbital fibroblasts were plated on 60-mm diameter dishes and allowed to reach confluence before being shifted to medium supplemented with 1% FBS with or without TGF-β or other test agents such as PMA for 6 h. The cells were then washed twice with ice-cold PBS, and the total cellular RNA isolated by the acid guanidinium-thiocyanate-phenol-chloroform extraction method [Chomczynski and Sacchhi, 1987]. For reverse transcription (RT), complementary DNA was synthesized using a First-strand cDNA Synthesis kit according to the manufacturer's protocol. RNA (20 μl, 5 μg) was added to a 13 μl reaction mixture containing 1 μl 0.2 μg/μl Pd $(N)_6$ primer, 1 μl 200 mM DTT solution, and 11 μl Bulk first-strand cDNA reaction mix. RT was carried out at 37°C for 60 min, then 90°C for 5 min and was terminated at 4°C.

For PCR amplification, 8 μl cDNA were added to a 42 μl reaction containing 5 μl 10× PCR buffer (500 mM KCl (pH 9.0), 15 mM $MgCl_2$, and 100 mM Tris-HCl), 31 μl ddH_2O, 1 μl 20 mM dNTPs, 2 μl sense and antisense primers (10 μM/μl), and 1 μl Taq DNA polymerase (5 U/μl). All the reagents were purchased for Amersham Pharmacia Biotech (NJ, USA). Reactions were carried out at 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and then 72°C for 7 min. Finally the reaction was terminated at 4°C. The product was analyzed on a 3% agarose gel. The oligonucleotide primers for HAS-1, HAS-2, HAS-3, and GAPDH were designed according to the published sequences [Nishida et al., 1999; Ohkawa et al., 1999]. The sequences were as follows: HAS-1 sense: 5′-TGG GGC GGC AAG CGC GAG GTC ATG TAC ACA GC-3′, antisense: 5′-CAC CAG AGC GCG TTG TAC AGC CAC TCA CGG AAG TA-3′ (the pair generated a 548 bp fragment);

HAS-2 sense: 5′-ATT GTT GGC TAC CAG TTT ATC CAA ACG G-3′, antisense: 5′-TTT CTT TAT GTG ACT CAT CTG TCT CAC CGG-3′ (the pair generated a 409 bp fragment); HAS-3 sense: 5′-AGA GAC CCC CAC TAA GTA CCT CCG-3′, antisense: 5′-CAG AAG GCT GGA CAT ATA GAG GAG GG-3′ (the pair generated a 331 bp fragment); GAPDH sense: 5′-CAC CAT CTT CCA GGA GCG AG-3′, antisense: 5′-TCA CGC CAC AAG TTT CCC GGA-3′ (the pair generated a 397 bp fragment).

Immunocytochemical Staining

Immunofluorescence staining was performed as previously described [Wu et al., 1996]. Cells cultured on coverslips were treated with PMA, TGF-β, or calphostin C for 1 h; cells were then treated with PMA or TGF-β or the other test agents for specific intervals. Cells were fixed with 10% formalin in PBS for 10 min at room temperature. After washing with PBS, the cells were blocked and permeabilized with 5% non-fat dry milk in PBS containing 0.1% (v/v) Triton X-100 for 15 min at room temperature. Cells were incubated with a 1:100 dilution of a rabbit anti-PKCα, anti-PKCβ, or anti-PKCγ antibodies for 1 h at 37°C, washed with PBS, and then incubated for 1 h at 37°C with a 1:50 dilution of FITC-conjugated, goat anti-rabbit IgG (Vector, Burlingame, CA). After washing with PBS, the cells were mounted using mounting medium (Vector, Burlingame, CA) and observed using a fluorescence microscope (Leica). In control samples in which the primary antibodies were omitted, negligible immunofluorescence was seen.

Statistical Analysis

Data were analyzed by one-way ANOVA. To compare means between groups, a protected Fisher's LSD (least significant difference) test was used at an alpha level of 0.05.

RESULTS

Effects of PKC and PKA Activation on TGF-β-Stimulated HA Synthesis

We investigated the possible role of PKC and PKA in TGF-β-induced HA synthesis in fibroblasts from various anatomical sites. The effects of TGF-β (10 ng/ml) and PMA (100 ng/ml) on HA synthesis were not additive. Forskolin (5 μg/ml) induced only a moderate stimulation of HA synthesis in normal orbital fibroblasts (Fig. 1A), fibroblasts from patients with Graves' ophthalmopathy (Fig. 1B), and abdominal fibroblasts (Fig. 1C). In addition, forskolin had an inhibitory effect on PMA- or TGF-β-induced HA synthesis. This supports the theory that PKC plays a major role in the activation of HAS in fibroblasts and suggest that there is cross-talk between the PKA and PKC pathways.

Effects of PKC Inhibition on HA Synthesis Stimulated by TGF-β1

To investigate whether the effects of TGF-β on HA synthesis were mediated via PKC activation, fibroblasts were pre-treated with the PKC inhibitor calphostin C and incubated with TGF-β. Calphostin C (10 ng/ml) caused a partial inhibition of TGF-β-induced HA synthesis (Fig. 2A–C). These results suggest that PKC played a role in TGF-β-induced stimulation of HA synthesis.

Effects of Cycloheximide on HA Production Stimulated by TGF-β1 and Kinase Activator

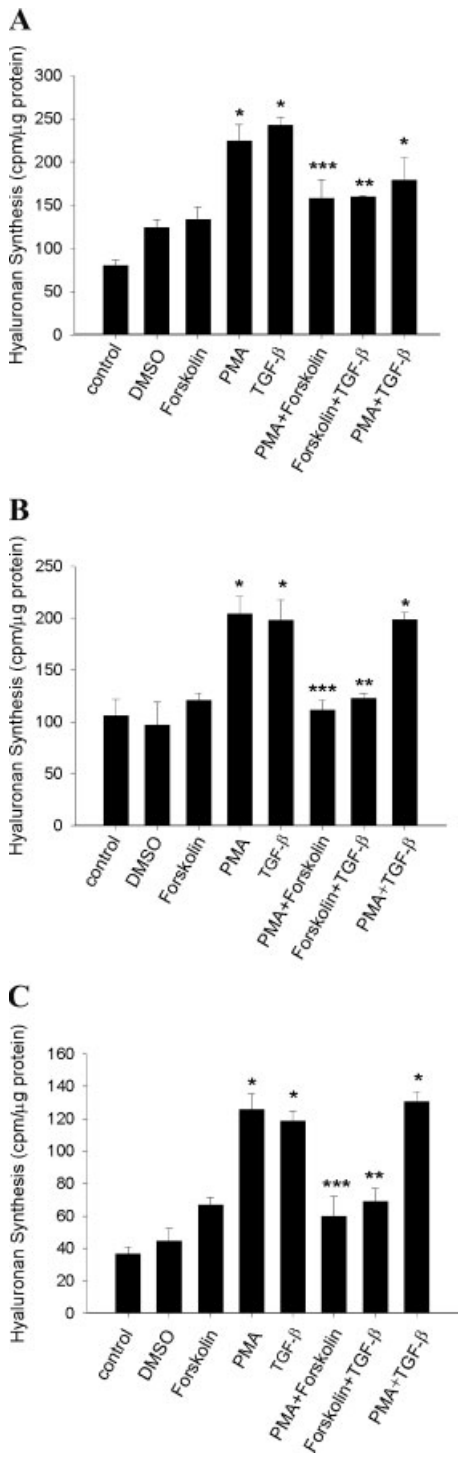
To determine whether the PMA- or TGF-β-induced HA production required new protein synthesis, cycloheximide, a protein synthesis inhibitor, was used. In the presence of cycloheximide, TGF-β-, or PMA-induced HA synthesis was completely (TGF-β) or almost completely (PMA) inhibited (Fig. 3A–C). Therefore, stimulation of HA synthesis by PKC and TGF-β involved new protein synthesis.

RT-PCR Analysis of HAS mRNA Expression in TGF-β Treated Orbital Fibroblasts

In order to test whether PMA- or TGF-β-induced HAS mRNA expression, RT-PCR analysis was used. As shown in Figure 4, the expression of the HAS isoform mRNAs varies. HAS1 appears to be expressed under both basal and PMA or TGF-β-treated conditions; there might be a slight increase in expression under the two treatments. In the case of HAS2, the mRNA is not present under basal culture conditions, but is induced by treatment with PMA or TGF-β for 6 h. HAS3 mRNA also could not be detected under basal condition, but was present after PMA or TGF-β treatment. The results showed that both PMA and TGF-β can induced hyaluronan synthase expression in orbital fibroblasts. This is consistent with the data described above where cycloheximide can inhibit TGF-β-induced HA synthesis.

Role of Intracellular Ca^{2+} in TGF-β-Induced HA Synthesis

An increase in the cytosolic free calcium concentration involves translocation of the Ca^{2+}-dependent PKC. To determine whether Ca^{2+} was required for TGF-β-induced HA synthesis, orbital fibroblasts were pre-loaded for 15 min with the Ca^{2+} chelator BAPTA (1 μM) and incubated with or without TGF-β for 24 h. The amount of HA produced was determined. The Ca^{2+} chelator partially inhibited TGF-β-stimulated HA synthesis, suggesting that intracellular Ca^{2+} is involved in this process (Fig. 5A–C).



Effects of Extracellular Calcium Concentration on HA Synthesis

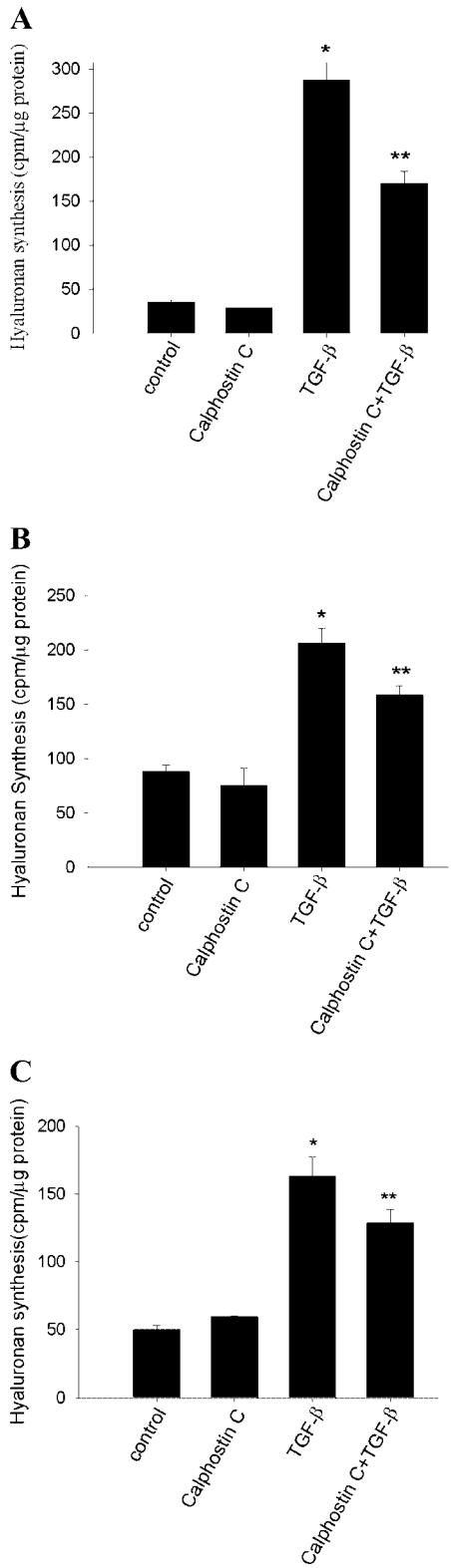
To verify whether calcium was required for HA synthesis, confluent fibroblasts were cultured in MEM supplemented with 1% FBS and with various calcium concentrations ranging from 0.05 to 4 mM. HA synthesis increased significantly in these fibroblasts as the calcium concentration was increased (Fig. 6A–C). The threshold of the stimulation occurred at or below 2.5 mM and appeared near maximal at 7.5 mM, where the increase in HA accumulation by calcium shows a biphasic dose response.

Effects of PKA Inhibition and PKC Activation on TGF-β-Induced HA Synthesis

Forskolin combined with PMA or TGF-β decreased HA synthesis in orbital fibroblasts (Fig. 1); therefore, we examined the possibility of cross-talk between the PKA and PKC pathways in HA synthesis. H-89, a PKA inhibitor, stimulated HA synthesis in orbital fibroblasts from patients with Graves' ophthalmopathy. In addition, H-89 combined with PMA or TGF-β enhanced HA synthesis (Fig. 7). This provided evidence for cross-talk between the PKA and PKC pathways in HA synthesis. The results suggested that PKA activation decreased HA synthesis in both control and stimulated cells. Therefore, both PKA and PKC influence HA

Fig. 1. Effects of TGF-β, PMA, and forskolin, alone or in combination, on hyaluronan (HA) synthesis in fibroblast cultures. Normal orbital fibroblasts (**A**), orbital fibroblasts from patients with Graves' ophthalmopathy (**B**), and abdominal fibroblasts (**C**) were allowed to proliferate to confluence. The medium containing 1% FBS was changed in all cultures, and the cells were cultured for 15 min with PMA (100 ng/ml), for 1 h with forskolin (5 μg/ml), either alone or in combination with TGF-β (10 ng/ml) for 48 h. Cultures were labeled with [^{3}H]glucosamine (1 μCi/ml) for the last 24 h and then harvested. Monolayers and media were combined and analyzed for total glycosaminoglycan (GAG) accumulation. HA synthesis was measured. Each data point represents the mean ± SEM of four determinations. $^*P < 0.05$ compared to control, $^{**}P < 0.05$ compared to TGF-β, $^{***}P < 0.05$ compared to PMA.

synthesis; specifically, PKA inhibits and PKC stimulates. The mechanisms are unclear, but the influences may be completely independent of each other.



Immunofluorescence Staining

The PKC isoforms are associated with the cytosolic proteins or with the cellular membrane. To characterize the cPKC isoforms expressed in orbital fibroblasts, the effects of TGF-β on PKC translocation were studied using immunofluorescence. In control cells, PKCα immunoreactivity was associated with the nucleus, and its intracellular distribution was not affected by treatment with TGF-β; similar results for the distribution and lack of effect of treatment were obtained for PKCγ (data not shown). The distribution of PKCβI immunoreactivity was not affected by treatment with PMA (data not shown). In contrast, PMA or TGF-β (Fig. 8C,E) induced an increase in the amount of PKCβII associated with the membrane compared to non-stimulated cells (Fig. 8A). Translocation was eliminated by calphostin C (Fig. 8D,F).

DISCUSSION

TGF-β is a potent inducer or modulator of a wide range of biological processes, including inhibition of growth, differentiation of several kinds of cells, and suppression of inflammation [Roberts et al., 1985; Sporn et al., 1987; Sharma and Ziyadeh, 1994]. Production of extracellular matrix components, such as type I and type III collagen, fibronectin, and proteoglycans also can be up-regulated by TGF-β in various cell types [Chen and Massague, 1999]. HA synthesis is stimulated by TGF-β in human lung fibroblasts but not in skin fibroblasts [Westergren-Thorsson et al., 1990]. In our experiments, HA synthesis in orbital fibroblasts was up-regulated by TGF-β.

In other studies, TGF-β has been shown to activate serine/threonine kinases [Massague, 1996; Massague and Weis-Garcia, 1996] and PKA [Wang et al., 1998]. The activation of HA

Fig. 2. Effect of calphostin C on TGF-β-induced HA synthesis. Confluent normal orbital fibroblasts (**A**), orbital fibroblasts from patients with Graves' ophthalmopathy (**B**), and abdominal fibroblasts (**C**) were incubated for 3 h in MEM containing 1% FBS, treated for 15 min with calphostin C (10 ng/ml), and incubated for 48 h at 37°C with TGF-β (10 ng/ml) in the continued presence of the inhibitors. For the last 24 h, [^{3}H]glucosamine (1 μCi/ml) were added to allow the labeling and measurement of HA synthesis. Values represent mean ± SEM. for four individual dishes. *$P < 0.05$ compared to control, **$P < 0.05$ compared to TGF-β.

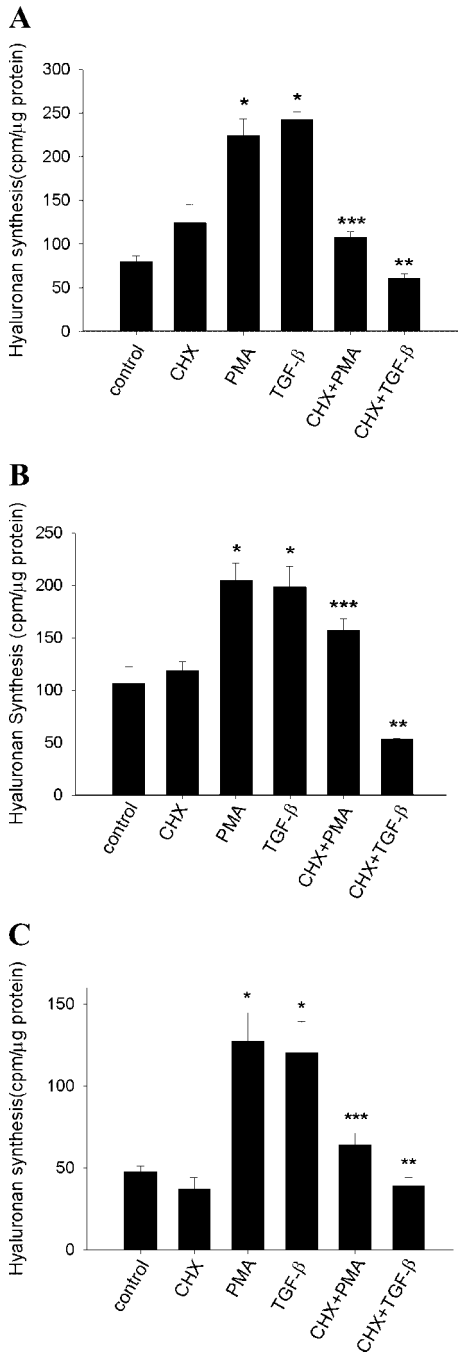


Fig. 3. Effect of cycloheximide on TGF-β- or PMA-induced HA synthesis. Normal orbital fibroblasts (**A**), orbital fibroblasts from patients with Graves' ophthalmopathy (**B**), and abdominal fibroblasts (**C**) were incubated with or without cycloheximide (10 μg/ml in 0.05% DMSO) for 30 min in MEM containing 1% FBS. The cells were then incubated with TGF-β (10 ng/ml), PMA (100 ng/ml), or medium for 48 h. Cultures were labeled with [^{3}H]glucosamine (1 μCi/ml) for the last 24 h and harvested. HA synthesis was then measured. *$P < 0.05$ compared to control, **$P < 0.05$ compared to TGF-β, ***$P < 0.05$ compared to PMA.

synthesis may involve PKC or the c-AMP-dependent protein kinases. We used distinct activators of these two kinases and this confirmed that PMA stimulated HA synthesis in fibroblasts. The effects of PMA and TGF-β were not additive, suggesting an important role for PKC in the up-regulation of HA synthesis in orbital fibroblasts. Addition of the PKC inhibitor calphostin C reduced HA synthesis induced by TGF-β. However, forskolin, an adenyl cyclase and PKA activator, did not affect HA synthesis. Interestingly, forskolin inhibited TGF-β-induced HA synthesis.

In other studies, inhibition of protein synthesis with cycloheximide decreased the rate of HA synthesis in 3T3 fibroblasts [Kitchen and Cysyk, 1995] and IL-1α-induced HA synthesis in orbital fibroblasts [Wong et al., 2001]. According to our results, TGF-β-stimulated HA production required protein synthesis, suggesting that induction of HAS mRNA and protein synthesis were required. By RT-PCR analysis, we found that both PMA- and TGF-β were able to significantly induce HAS2 mRNA and HAS3 mRNA expression (Fig. 4). In contrast, HAS1 mRNA was present under basal conditions and was not affected by PMA- and TGF-β.

Phosphatidylcholine-phospholipase C (PC-PLC) and PKC are involved in TGF-β signaling [Halstead and Ignotz, 1995]. PLC activation may increase levels of inositol 1, 4, 5-triphosphate and DAG; an increase in the cytosolic free calcium concentration ($[Ca^{2+}]_i$) follows. In the present study, TGF-β-induced HA synthesis was completely blocked in fibroblasts pretreated with BAPTA. TGF-β may act through a Ca^{2+}-sensitive PKC (a cPKC isoform) to induce HA synthesis in orbital fibroblasts. cPKC is a Ca^{2+}-dependent signaling intermediary that can be activated by an increase in $[Ca^{2+}]_i$. Since, Ca^{2+} activates cPKCs and PMA increases HA synthesis, we explored the potential role of cPKC in TGF-β-induced HA synthesis. cPKCs were depleted from orbital fibroblasts by an overnight PMA treatment; HA synthesis was blocked in these fibroblasts. PMA, which causes cPKC down-regulation, eliminates all cPKC isoforms except for the atypical PKC; therefore, TGF-β-induced HA synthesis must occur via cPKC activation.

Calcium chelation was more effective than PKC inhibition in reducing TGF-β-induced HA synthesis. An increase in the extracellular

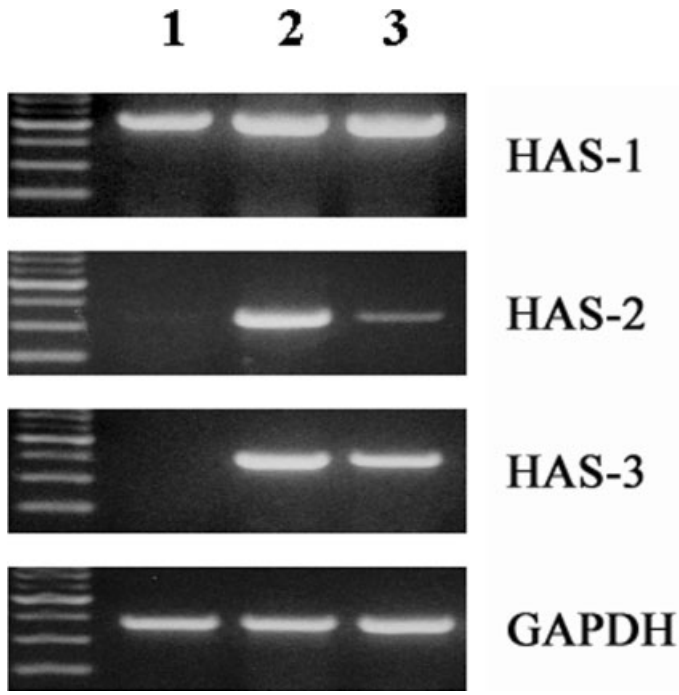


Fig. 4. RT-PCR analysis of HAS expression in orbital fibroblasts from patients with Graves' ophthalmopathy. Orbital fibroblasts from patients with Graves' ophthalmopathy were cultured in 100-mm diameter culture plates in medium containing 10% FBS. They were then shifted to medium with 1% FBS without or with TGF-β for 6 h. The monolayers were washed in PBS, then the monolayers were harvested, and the cellular RNA was extracted. The RNA was subjected to RT-PCR for the HAS transcripts as described in Materials and Methods. The products were subjected to electrophoresis and ethidium bromide staining and were visualized under UV light. **Lane 1**, control; **lane 2**, PMA treated; **lane 3**, TGF-β treated.

calcium concentration resulted in increased HA synthesis. These results are consistent with our previous study in which HA synthesis was induced by a calcium ionophore (A23187) that caused Ca^{2+} influx [Wong et al., 2001]. However, little is known about the mechanism by which calcium elicits this response.

PKC and PKA signal transduction pathways may be involved in activating HA synthesis [Honda et al., 1993; Imai et al., 1994; Klews and Prehm, 1994; Suzuki et al., 1995]. The effects of the PKA inhibitor H-89 on HA synthesis in fibroblasts treated with TGF-β and PMA are particularly interesting. H-89 alone or H-89 combined with PMA or TGF-β are able to up-regulated HA synthesis. Our data suggested that there was cross-talk between PKA and PKC in HA synthesis and in TGF-β-induced HA synthesis. Under basal conditions, blocking of PKA increases HA synthesis by about nine-fold and TGF-β or PMA-stimulation increases this only slightly above the PKA-blocked effect (Fig. 7). Furthermore, blocking of PKC in TGF-β stimulated cells decreases HA synthesis by only 15%–30% (Fig. 2). One possible interpretation of this is that the major effect of TGF-β or PMA on HA synthesis is by inhibition of PKA with a smaller contribution from PKC stimulation.

PKC activation was observed to occur by the translocation of PKC from the cytosol to the cellular membrane. The immunocytochemical localization of PKCα, PKCγ, and PKCβI remained unchanged following PMA or TGF-β stimulation. In contrast, translocation of PKCβII to the membrane was seen following TGF-β treatment. The localization of PKCβII immunoreactivity in TGF-β-stimulated cells was altered by calphostin C (Fig. 8). Apparently, PKCβII was the isoform involved in TGF-β-induced activation of the PKC pathway in orbital fibroblasts.

Since after TGF-β treatment, the HA synthesis in orbital fibroblasts is about two-fold higher than that in fibroblasts from the abdomen, we suggest that the local microenvironment and the milieu of agonists and antagonists that the fibroblast encounters, plus the developmental propensities of the connective tissue in certain body regions, are all involved in

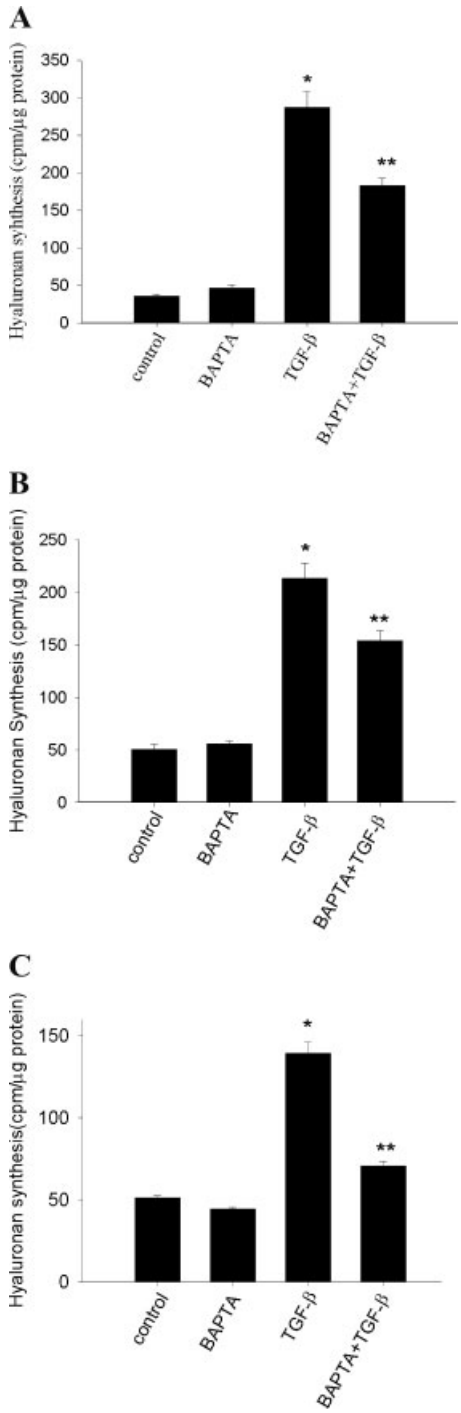


Fig. 5. Effects of TGF-β and BAPTA, alone or in combination, on HA synthesis. Confluent normal orbital fibroblasts (**A**), orbital fibroblasts from patients with Graves' ophthalmopathy (**B**), and abdominal fibroblasts (**C**) were incubated for 15 min in the presence or absence of BAPTA (1 μM). The cells were then incubated for 24 h with or without TGF-β (10 ng/ml). The amount of HA synthesized was determined. The values represent the mean ± SEM. for four individual dishes. $*P < 0.05$ compared to control, $**P < 0.05$ compared to TGF-β.

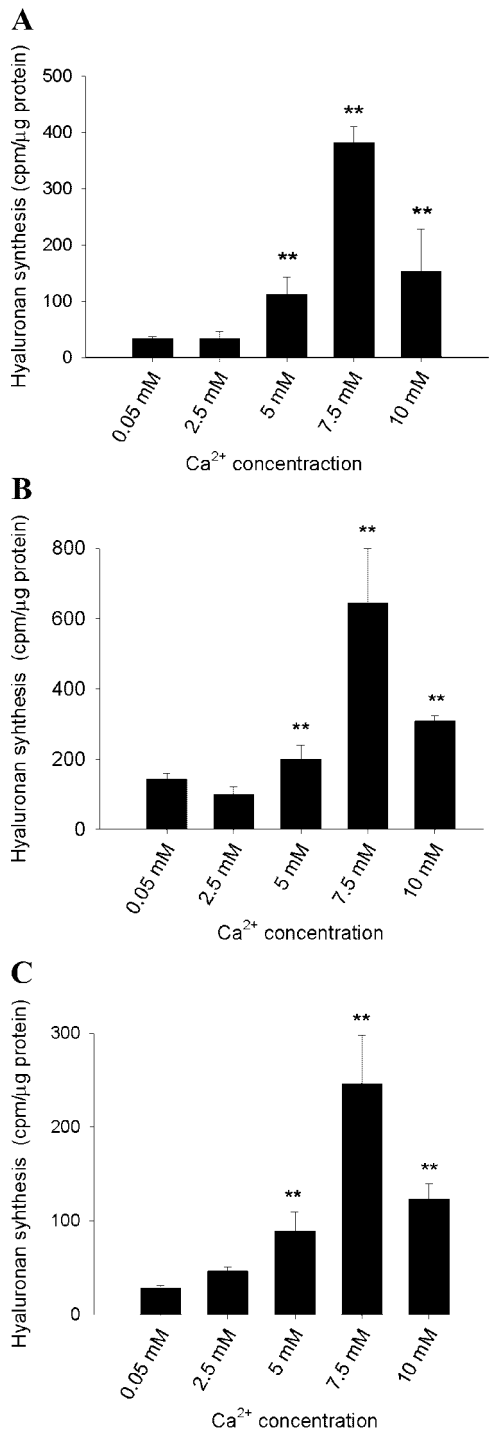


Fig. 6. Effects of increased calcium concentrations on HA synthesis in cultured fibroblasts. Confluent normal orbital fibroblasts (**A**), orbital fibroblasts from patients with Graves' ophthalmopathy (**B**), and abdominal fibroblasts (**C**) were cultured for 24 h in MEM supplemented with 1% FBS and the indicated concentration of calcium. The cells were then labeled with [^{3}H] glucosamine (1 μCi/ml). The medium and cell layers were combined and assayed for HA synthesis. Each data point represents the mean ± SEM for four determinations. $**P < 0.05$ compared to control.

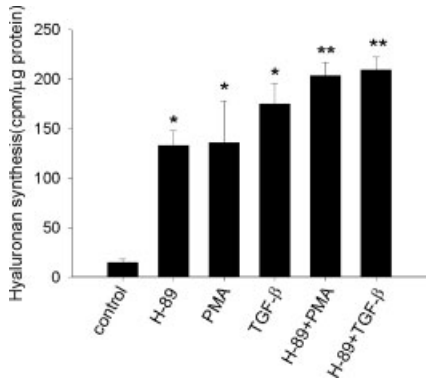


Fig. 7. Effects of TGF-β, PMA, and H-89, alone or in combination, on HA synthesis in orbital fibroblast cultures. Orbital fibroblasts from patients with Graves' ophthalmopathy were allowed to proliferate to confluence. Medium was replaced with MEM containing 1% FBS in all cultures, and the cells were cultured for 15 min with PMA (100 ng/ml) or for 1 h with H-89 (10 ng/ml), either alone or in combination with TGF-β (10 ng/ml) for 24 h. Cultures were then labeled with [^{3}H]glucosamine (1 μCi/ml) for 24 h and harvested. Monolayers and media were combined and analyzed for total GAG accumulation. HA synthesis was measured. Each data point represents the mean ± SEM of four determinations. $^*P < 0.05$ compared to control, $^{**}P < 0.05$ compared to TGF-β.

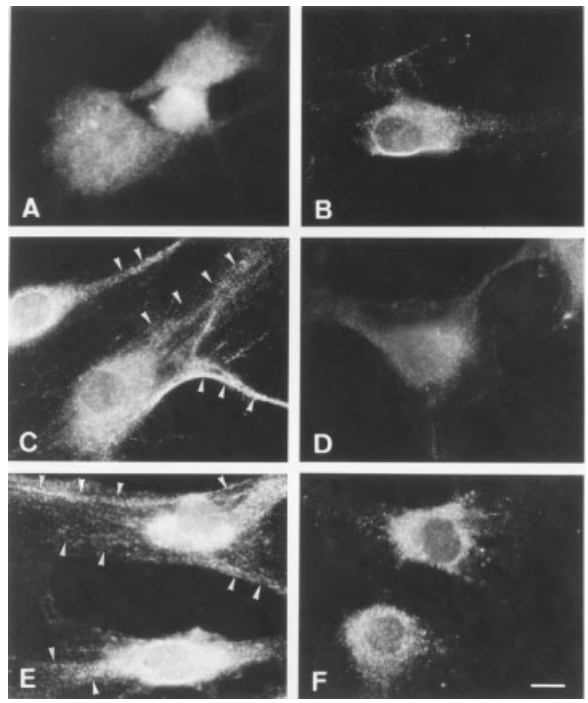


Fig. 8. Immunocytochemical localization of PKCβII in orbital fibroblasts from patients with Graves' ophthalmopathy. Orbital fibroblasts from patients with Graves' ophthalmopathy seeded on coverslips were stained for immunofluorescence with anti-PKCβII antibody. **A**: Control orbital fibroblasts. **B**: Fibroblasts treated for 1 h with calphostin C (100 ng/ml). **C**: Cells incubated with PMA (100 ng/ml) for 15 min before immunofluorescence staining. **D**: Cells pre-treated for 1 h with calphostin C and incubated with PMA for 15 min. **E**: Cells treated for 30 min with TGF-β. **F**: Cells pre-treated for 1 h with calphostin C and then stimulated with TGF-β for 30 min. In (C) and (F), PKCβII was translocated to the cell membrane (arrow heads). In (D) and (F), calphostin C inhibited PKCβII translocation. The bar corresponds to 10 μm.

localized manifestations of systemic disease. The increased amounts of HA evident in the retroocular space of patients with Graves' ophthalmopathy are consistent with this scenario [Hufnagel et al., 1984]. TGF-β-induced HA synthesis is mediated by PKCβII in fibroblasts. Orbital fibroblasts, together with macrophages and other immune cells, may contribute to increased cytokine levels. TGF-β may participate in the inflammatory events occurring in the orbit and consequently play an important role in the pathogenesis of Graves' ophthalmopathy. Because PKCβII is a key enzyme in the regulation of HA synthesis by TGF-β, this enzyme might be an appropriate target for therapeutic compounds to be used to treat Graves' ophthalmopathy accompanied by inflammation.

In summary, we have provided evidence that TGF-β induced HA synthesis and that cPKC was involved in this process. The effects of TGF-β and PMA on HA synthesis were dependent on the synthesis of new proteins. TGF-β had no tissue-specific effects on matrix production; therefore, overall HA production in orbital fibroblasts was most likely to be regulated by PKCβII. Cross talking was evident between PKA and PKC in HA synthesis and in TGF-β-induced HA synthesis. PKC activation and calcium influx were necessary and sufficient for TGF-β-induced HA synthesis in fibroblasts.

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