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Stimulation of Hyaluronan Synthesis by Interleukin-1β Involves Activation of Protein Kinase C βII in Fibroblasts From Patients with Graves' Ophthalmopathy

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Abstract Hyaluronan accumulation in the retroorbital connective tissue is one of the pathological features of Graves' ophthalmopathy. Interleukin-1β (IL-1β) is known to stimulate hyaluronan synthesis in orbital fibroblasts. In the present study, the intracellular signal transduction pathways involved in this stimulatory effect were investigated in cultured human retroorbital fibroblasts from patients with Graves' ophthalmopathy. IL-1β-induced hyaluronan synthesis was significantly inhibited by pretreatment of the cells with two protein kinase C (PKC) inhibitors, chlerythrine chloride and H-7. In addition, treatment with phorbol 12-myristate 13-acetate (PMA), a direct PKC activator, also resulted in increased hyaluronan production. IL-1β- or PMA-stimulated hyaluronan synthesis was blocked by the protein synthesis inhibitor, cycloheximide. Moreover, the intracellular Ca^{2+} concentration of the orbital fibroblasts was also involved in the IL-1β induced transduction pathway, the effect being completely inhibited by BAPTA, an internal calcium chelator. In addition, A23187, a calcium ionophore, increased hyaluronan synthesis in unstimulated cells. These results suggest that the Ca^{2+}-dependent PKC signal transduction pathway plays an important role in the IL-1β-induced hyaluronan synthesis. Moreover, IL-1β treatment resulted in increased PKC activity and the rapid translocation of PKC βII from the cytoplasm to the plasma membrane. These results indicate that cytosolic Ca^{2+} and PKC βII are involved in IL-1β-induced hyaluronan synthesis in cultured orbital fibroblasts from patients with Graves' ophthalmopathy. J. Cell. Biochem. 82: 58–67, 2001.

Key words: hyaluronan; interleukin-1β; orbital fibroblast; Graves' ophthalmopathy

Hyaluronan, a linear polysaccharide composed of glucuronic acid and N-acetyl-glucosamine, is a characteristic component of the extracellular matrix and provides cellular support and regulates cell to 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].

Graves' ophthalmopathy is an autoimmune disease characterized by infiltration of the retroocular space by lymphocytes, macrophages, and mast cells, and by an inflammatory response [Bahn and Heufelder, 1992]. Large increases in the amounts of hyaluronan in the retroocular space are seen in patients with Graves' ophthalmopathy [Hufnagel et al., 1984]. There is evidence that pro-inflammatory cytokines are involved in the pathogenesis of Graves' ophthalmopathy [Bahn and Heufelder, 1993; Heufelder, 1995; Bartalena et al., 1996; Ajjan et al., 1997]. A variety of inflammatory mediators, including interferon-γ (IFN-γ), interleukin-1 (IL-1), and transforming growth factor-β (TGF-β), have been shown to stimulate glycosaminoglycan (GAG) synthesis in cultured human retroorbital fibroblasts [Campbell, 1984; Smith et al., 1991; Imai et al., 1992; Korducki et al., 1992]. Furthermore, accumulation of

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GAG (predominantly hyaluronan) within the perimysial and retroocular connective tissue increases the volume of tissue in the orbit, leading to proptosis, diplopia, periorbital swelling, and inflammation [Smith et al., 1989; Bahn and Heufelder, 1992]. Glucocorticoid treatment of cultured retroocular fibroblasts inhibits cytokine-induced hyaluronan synthesis [Smith, 1984; Bahn and Heufelder, 1992]. Normal fibroblasts and those from patients with Graves ophthalmopathy respond similarly in terms of GAG synthesis in response to IL-1 stimulation [Korducki et al., 1992]. However, fibroblasts derived from different anatomical regions appear to differ in their biological responses to cytokines, including changes in cell morphology [Wang et al., 1996], hyaluronan synthesis [Smith et al., 1995], and specific protein synthesis [Cao et al., 1998]. Thus, the different physiological roles of fibroblasts in different regions of the body might explain the propensity of the connective tissue investing certain body regions to become involved in localized manifestations of systemic diseases. Taken together, these findings suggest that locally acting cytokines stimulate GAG production in fibroblasts, leading to GAG accumulation in the orbital connective tissue, which play an important role in the clinical manifestations of Graves' ophthalmopathy.

IL-1β is a pro-inflammatory cytokine that affects most cell types, triggering a variety of responses. When IL-1β binds to its cell-surface receptor, it initiates a signaling cascade that subsequently leads to activation of the transcription factor, NF-κB, and is relayed through TNF receptor-associated factor 6 (TRAF6) and a succession of kinase enzymes, including NF-κB-inducing kinase (NIK) and IκB kinases (IKKs) [Muzio et al., 1997]. However, the molecular mechanism by which NIK is activated is not understood.

PKC is a family of serine/threonine kinases which play crucial roles in various signaling processes, including proliferation and differentiation. Molecular cloning studies have shown that this family consists of at least 12 distinct members divided into three subgroups, the conventional (cPKC), novel (nPKC), and atypical (aPKC) isoforms [Nishizuka, 1995]. cPKCs require Ca^{2+} and are activated by phosphatidylserine (PS), diacylglycerol (DAG), or PMA, whereas nPKCs are activated by PS, DAG, or PMA, but not by Ca^{2+}, and aPKCs are activated by PS, but not by Ca^{2+}, DAG, or PMA [Berry and Nishizuka, 1990; Hug and Sarre, 1993; Dekker and Parker, 1994, Newton, 1995].

While IL-1β is an important mediator of hyaluronan synthesis in fibroblasts [Cao et al., 1998], the mechanism by which it mediates signal transduction in orbital fibroblasts is poorly understood. The aim of the present study was to determine, using a variety of approaches (inhibitors, activator analysis, and Ca^{2+} chelation), whether IL-β-induced hyaluronan synthesis in orbital fibroblasts from Graves' ophthalmopathy patients is mediated by PKC and Ca^{2+}. The results show that this process involves an increase in the internal Ca^{2+} concentration $[Ca^{2+}]i$ and activation of protein kinase C βII.

MATERIALS AND METHODS

Cell Culture

Human orbital fibroblasts from patients with Graves' ophthalmopathy who had undergone decompression surgery were provided by Dr. T. J. Smith (Albany Medical Center, NY). These cells were grown in minimum essential medium (MEM) (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (FBS), penicillin/streptomycin, nystatin, and glutamine in a 5% CO_2 humidified incubator at 37°C, the medium being changed every 3–4 days. Cells were used before passage 12.

[^{3}H]Glycosaminoglycan Assay

For tests, cells were plated in 60 mm diameter plastic dishes and allowed to reach confluency before being shifted for the indicated time periods to medium supplemented with 1% FBS with or without recombinant IL-1β (R&D, Minneapolis, MN) or other test agents, such as PMA, cycloheximide, and forskolin (Sigma, St. Louis, MO). When used, the PKC inhibitors, H-7 and chelerythrine chloride (Calbiochem, San Diego, CA), were added 1 h before any other treatment. The cultures were then labeled for 16 h with [^{3}H]glucosamine (1 μCi/ml) (DuPont-NEN Life Science, Boston, MA). At the time of cell harvest, the medium was removed, the cell layer rinsed with phosphate-buffered saline (PBS), and the PBS rinse pooled with the medium sample. The cell layer was removed from the substratum using 2 ml of 0.2 N NaOH and a rubber policeman, and the cells disrupted by sonication. Quantification of radiolabeled

GAG was performed as described by Smith, 1984. Briefly, an aliquot of the solubilized cell layer was taken for cell protein quantification using the BCA protein assay kit (Pierce, Rockford, Illinois), then the medium and the remainder of the solubilized cell layer were combined and subjected to pronase (1 mg/ml) digestion at 50°C overnight in 100 mM Tris (hydroxymethyl) aminomethane buffer, pH 8.0. The samples were then cooled to 4°C, and trichloroacetic acid added to a final concentration of 5% (w/v). After incubation for 1 h on ice, the samples were centrifuged and the acid-soluble material dialyzed against cold water. The retained GAG were then counted by liquid scintillation [Smith, 1984].

Intracellular Calcium Concentration ($[Ca^{2+}]_i$)

The AM ester of fura-2 (fura-2/AM) is a UV-excitable, ratiometric Ca^{2+} indicator that can passively diffuse across the cell membrane. To measure the $[Ca^{2+}]_i$, human orbital fibroblasts, cultured in MEM containing 10% FBS, were preloaded for 45 min at 37°C with 5 μM fura-2/AM (Molecular Probes, Inc., Eugene, OR, U.S.A.), washed twice, and resuspended at a concentration of 1×10^6 cells/ml in Hank's buffered saline solution (HBSS). After drug treatment for 10 min, 2 ml of each cell suspension was transferred to individual cuvettes and gently mixed at 37°C for 3 min using a micromagnetic stirrer. The fluorescence of the fura-2-loaded cells was measured on a spectrophotometer (Hitachi F-4500) with excitation at 340 and 380 nm and emission at 510 nm. The intracellular calcium concentration for each sample was calculated from the emission/excitation ratios according to the equation derived by Grynkiewicz et al., 1985.

$$[Ca^{2+}]_i = K \cdot (R - R_{min})(S_{f380})/(R_{max} - R)(S_{b380})$$

where K = 224 nM (Fura-2 at 37°C), R = the emission/excitation ratio at the Ca^{2+} concentration in the medium, R_{min} = the ratio in minimal Ca^{2+} conditions, R_{max} = the ratio at the maximal Ca^{2+} concentration, S_{f380} = the 380 nm reading in minimal conditions (corrected for background), and S_{b380} = the 380 nm reading at the maximal Ca^{2+} conditions (corrected for background). The values for R_{max} and S_{b380} were obtained at the end of each measurement by permeabilizing the cells with 0.2% digitonin, while those for R_{min} and S_{f380} were determined by adding 20 mM EGTA after digitonin lysis. All measurements were performed in Ca^{2+}-containing medium, since no significant changes in $[Ca^{2+}]_i$ could be detected under Ca^{2+}-free conditions.

Measurement of PKC Activity

The cytosolic fraction of orbital fibroblast cells was assayed for PKC activity, determined by measuring the incorporation of ^{32}P from [γ-^{32}P]-ATP (DuPont-NEN) into a specific peptide substrate of PKC, supplied with the PKC assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, fibroblasts were pelleted, resuspended in lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 50 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml of leupeptin, 1 mM NaF, 1 mM Na_3VO_4, 2 mM EDTA, and 5 mM EGTA, and sonicated on ice. After centrifugation at 100,000 × *g* at 4°C for 60 min, the supernatant (cytosolic fraction) containing soluble PKC was collected, and its PKC activity measured using the PKC assay kit following the manufacturer's instructions. The results were normalized for protein concentration, determined using a BCA protein assay kit.

Immunohistochemical Staining

Immunofluorescence staining was performed as previously described [Wu et al., 1996]. Cells, cultured on coverslips, were treated with PMA or IL-1β, then fixed for 10 min at room temperature with 10% formalin in PBS. After washing with PBS, the cells were blocked and permeabilized by treatment for 15 min at room temperature with 5% nonfat dry milk in PBS containing 0.1% (v/v) Triton X-100. They were then incubated for 1 h at 37°C with a 1:100 dilution of a rabbit anti-PKCα, anti-PKCβII, or anti-PKCγ antiserum (all from Gibco), washed with PBS, 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 Vectashield mounting medium (Vector, Burlingame CA) and viewed with a Leica confocal laser scanning microscope using a 100X/1.30 oil immersion objective and an appropriate filter. In controls in which the primary antibodies were omitted, negligible immunofluorescence was seen.

Statistical Analysis

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

RESULTS

Effect of Increasing Concentrations of IL-1β on Hyaluronan Accumulation in Orbital Fibroblast Cultures

Under basal culture conditions, orbital fibroblasts synthesize hyaluronan, as shown by the incorporation of [^{3}H]glucosamine into macromolecular material. When fibroblast monolayers were treated with IL-1β for 48 h of which the final 24 h were in the presence of [^{3}H]glucosamine, [^{3}H]hyaluronan production was increased. The dose-response curve (Fig. 1) showed stimulation even at the lowest concentration of IL-1β tested (2.5 U/ml) and was near-maximal at 25 U/ml, with an approximately 2.3-fold increase in [^{3}H]glucosamine incorporation into GAG compared with controls (one-way ANOVA, LSD test, $P < 0.05$). PMA (100 ng/ml) also significantly increased hyaluronan synthesis (Fig. 2).

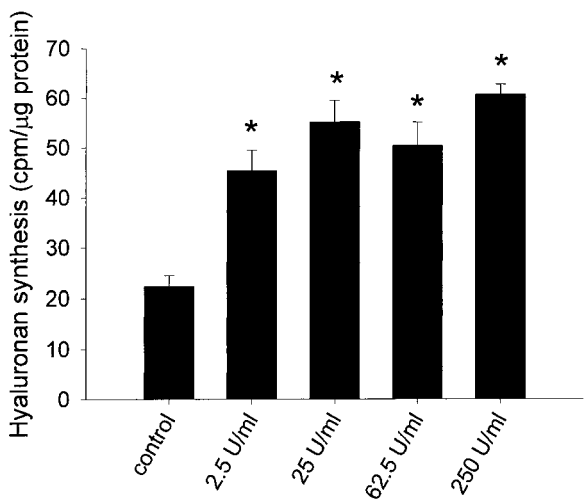

Fig. 1. Effect of increasing concentrations of IL-1β on hyaluronan synthesis in cultured orbital fibroblast cultures. Confluent cells were shifted for 24 h to MEM supplemented with 1% FBS and the indicated concentrations of IL-1β, then labeled with [^{3}H]glucosamine (1 μCi/ml). The medium and cell layers were combined and assayed for hyaluronan as described in the Materials and Methods. Each data point represents the mean ±SEM for quadruplicate determinations. *, $P < 0.05$ compared to control.

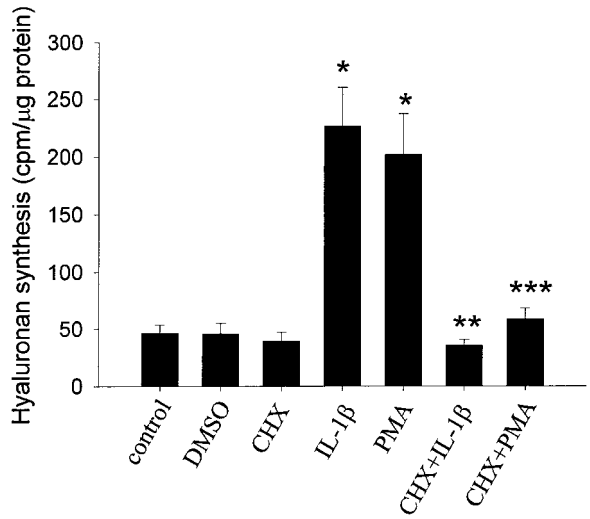

Fig. 2. Effect of cycloheximide on IL-1β- or PMA-induced hyaluronan synthesis. Orbital fibroblasts were incubated with or without cycloheximide (CHX; 10 μg/ml in 0.05% DMSO) for 30 min in medium containing 1% FBS, then with IL-1β (25 U/ml), PMA (100 ng/ml), or medium for 48 h. The amount of hyaluronan produced was then measured. Each data point represents the mean ± SEM for quadruplicate plates from a representative experiment. *, $P < 0.05$ compared to control. **, $P < 0.05$ compared to IL-1β, ***, $P < 0.05$ compared to PMA.

Effect of a Protein Synthesis Inhibitor on Hyaluronan Synthesis Stimulated by IL-1β and Kinase Activators

To determine whether these effects required new protein synthesis, cycloheximide, a protein synthesis inhibitor, was used and was found to significantly attenuate IL-1β- or PMA-induced hyaluronan synthesis (Fig. 2), suggesting that stimulation of hyaluronan synthesis by PKC or IL-1 β involves new synthesis of hyaluronan synthase or hyaluronan synthase regulators.

Effect of Activation of PKC and PKA on IL-1β-Stimulated Hyaluronan Synthesis

IL-1β is known to stimulate hyaluronan synthesis in human fibroblasts [Cao et al., 1998], as does PMA, a PKC activator, in human foreskin fibroblasts [Suzuki et al., 1995]. In addition, forskolin, a PKA activator, stimulates hyaluronan synthesis and hyaluronan synthase activity in rabbit pericardial mesothelial cells [Honda et al., 1993]. We therefore investigated the possible roles of PKC and PKA in IL-1β-induced hyaluronan biosynthesis in orbital fibroblasts. The results, presented in Figure 3, show that the effects of IL-1β (25 U/ml) and PMA (100 ng/ml) were not additive and that

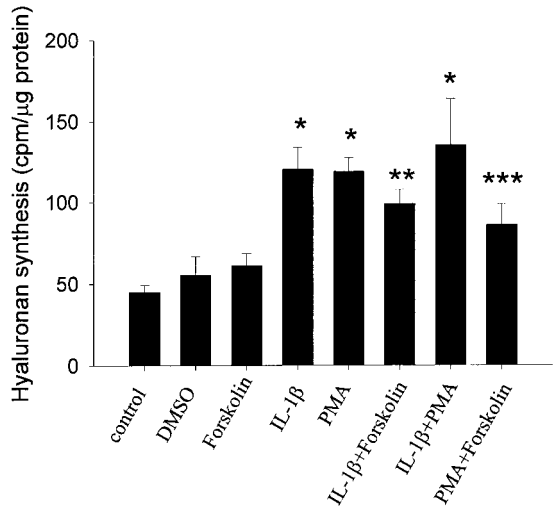

Fig. 3. Effects of IL-1β, PMA, and forskolin, alone or in combination, on hyaluronan synthesis in orbital fibroblast cultures. The amounts of hyaluronan produced was measured after 48 h of stimulation with IL-1β (25 U/ml), PMA (100 ng/ml), or forskolin (5 μg/ml), either alone or in combination. Hyaluronan synthesis was measured as described in the Materials and Methods. Data are expressed as the mean±SEM for quadruplicate cultures from a representative experiment. *, $P < 0.05$ compared to control; **, $P < 0.05$ compared to IL-1β; ***, $P < 0.05$ compared to PMA.

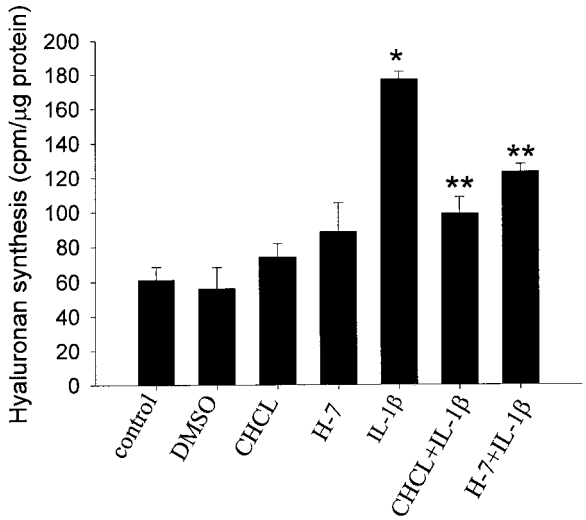

Fig. 4. Stimulation of hyaluronan synthesis by IL-1β effect of PKC inhibitors. Confluent orbital fibroblasts were preincubated for 3 h in MEM containing 1% FBS, then pretreated for 15 min with or without the PKC inhibitors, chelerythrine chloride (CHCL; 1 μg/ml) or H-7 (20 μg/ml), before a 48 h incubation at 37°C with or without IL-1β (25 U/ml) in the continued presence of the inhibitors. Hyaluronan synthesis was measured as described in the Materials and Methods. The values represent expressed as the mean±SEM for quadruplicate dishes from a representative experiment. *, $P < 0.05$ compared to control; **, $P < 0.05$ compared to IL-1β.

forskolin (5 μg/ml) only induced moderate stimulation of hyaluronan production. In addition, forskolin had an inhibitory effect on PMA- or IL-1β-induced hyaluronan synthesis. These data support the idea that PKC, but not PKA, plays a major role in the activation of hyaluronan synthase.

PKC Involvement in Stimulation ofHyaluronan Synthesis by IL-1β

In order to investigate whether the effects of IL-1β on hyaluronan synthesis were mediated via PKC activation, fibroblasts were pretreated with the PKC inhibitors, H-7 or chelerythrine chloride, and subsequently incubated with IL-1β. As shown in Figure 4, chelerythrine chloride (1 μg/ml) inhibited most of the IL-1β-induced hyaluronan synthesis, while H-7 (20 μg/ml) caused partial inhibition. These results suggest that PKC plays an important role in IL-1β-induced stimulation of hyaluronan synthesis.

Role of Intracellular Ca^{2+} in IL-1β-Induced Hyaluronan Synthesis

A $[Ca^{2+}]_i$ rise triggers the activation of a variety of cellular proteins, including Ca^{2+}-dependent cPKCs (reviewed by Nishizuka, 1995). To determine whether Ca^{2+} was required for IL-1β-induced activation of hyaluronan synthesis, orbital fibroblasts were pre-loaded for 15 min with the Ca^{2+} chelator, 1,2-bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid, (BAPTA-AM; 1 μM), then incubated with or without IL-1β for 24 h and the amount of hyaluronan produced determined. As shown in Figure 5A, the Ca^{2+} chelator significantly inhibited IL-1β-stimulated hyaluronan synthesis, suggesting that Ca^{2+} is involved in this process. For further analysis, we used a calcium ionophore, A23187, to examine the effect of intracellular calcium changes on hyaluronan synthesis. As shown in Figure 5B, addition of 5 μg/ml of A23187 resulted in a significant increase in hyaluronan synthesis. These results show that changes in the $[Ca^{2+}]_i$ can directly influence hyaluronan synthesis and are involved in IL-β induced hyaluronan synthesis.

PMA and IL-1β Induce an Increase in the $[Ca^{2+}]_i$

To determine whether IL-1β induces a change in the $[Ca^{2+}]$i, orbital fibroblasts were preloaded with 5 μM fura-2/AM and resuspended at a concentration of 1×10^6 cells/ml in HBSS, then

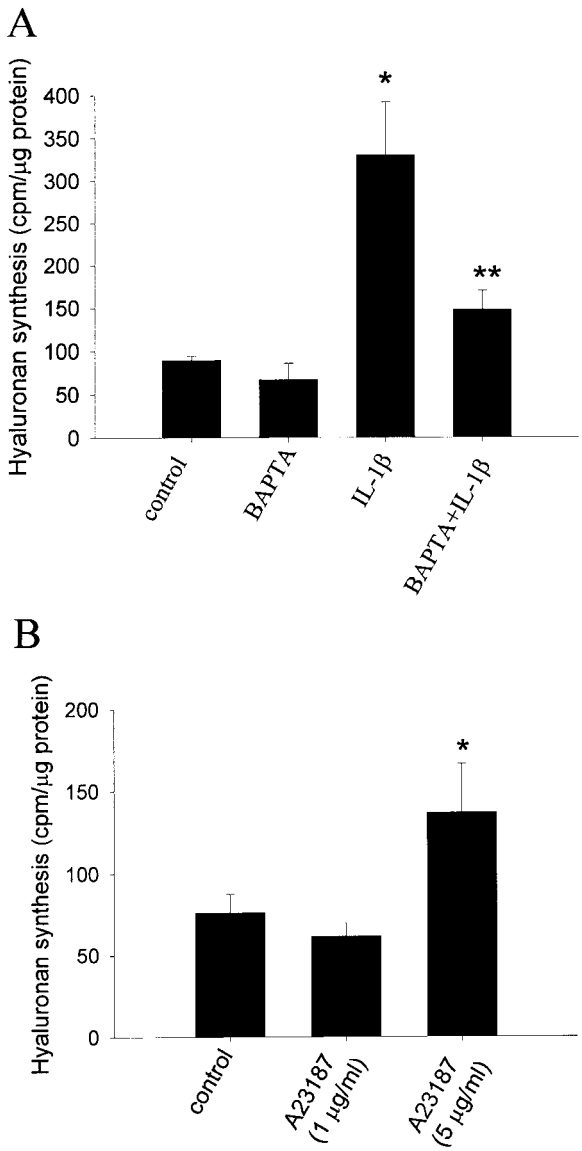

Fig. 5. Effect of BAPTA or A23187 on hyaluronan synthesis. **A**: Orbital fibroblasts were incubated for 15 min in the presence or absence of BAPTA (1 μM), then incubated for 24 h with or without IL-1β (25 U/ml). **B**: Orbital fibroblasts were incubated with A23187 (1 or 5 μg/ml) for 1 h. The amount of hyaluronan released into the media was determined as described in the Materials and Methods. The values represent the mean±S.E.M. for four individual dishes from a representative experiment. *, $P < 0.05$ compared to control, **, $P < 0.05$ compared to IL-1β.

their fluorescence was measured on a spectrophotometer (Hitachi F-4500) with excitation at 340 and 380 nm and emission at 510 nm before, and after, addition of IL-1β or PMA. The results, presented in Figure 6, show that both PMA and IL-1β induced a significant increase in the $[Ca^{2+}]i$, with PMA (100 ng/ml) inducing a net increase of 33.00±7.60 nM and IL-1β (25 U/ml) a net increase of 46.33±4.98 nM.

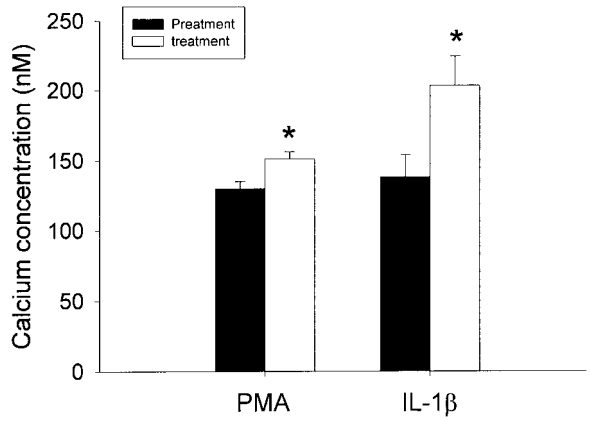

Fig. 6. PMA and IL-1β induce $[Ca^{2+}]i$ changes. Cells were preloaded with 5 μM fura-2/AM and resuspended in HBSS. The intracellular calcium concentration of each sample was measured on a spectrophotometer before, and after, addition of PMA (10 ng/ml) or IL-1β (25 U/ml). The values are the mean±SEM for 3–4 experiments. The data were analyzed by univariate analysis using the SAS program (*, $P < 0.001$).

Effect of IL-1β on PKC Activity

We then studied the effect of IL-1β on PKC activity in orbital fibroblast lysates. The results, presented in Table I, show that the PKC activity in plasma membrane fractions from IL-1β-stimulated cells was greater than that in membranes from unstimulated cells.

Immunofluorescence Microscopy

PKC isoforms are found in association with cytosolic proteins or the cellular membrane. To characterize the cPKC isoforms expressed in orbital fibroblasts, the effects of PMA and IL-1β on PKC translocation were studied by immunofluorescence experiments. In untreated cells, PKCα and PKCγ immunoreactivity was associated with the nucleus, and their intracellular distribution was not affected by treatment with either IL-1β or PMA (data not shown). In contrast, PKC βII was found in the cytoplasm

TABLE I. Effect of IL-1β Treatment of Cells on PKC Activity in the Plasma Membrane

	PKC activity pmol ^{32}P incorporated/min/μg protein
Control cells	107.3±5.7
IL-1β (25 U/ml)-treated cells	501.1*±101

All values are expressed as the mean ±SEM of triplicate experiments. To compare means between groups, a protected Fisher's LSD test was used.
*$P < 0.05$.

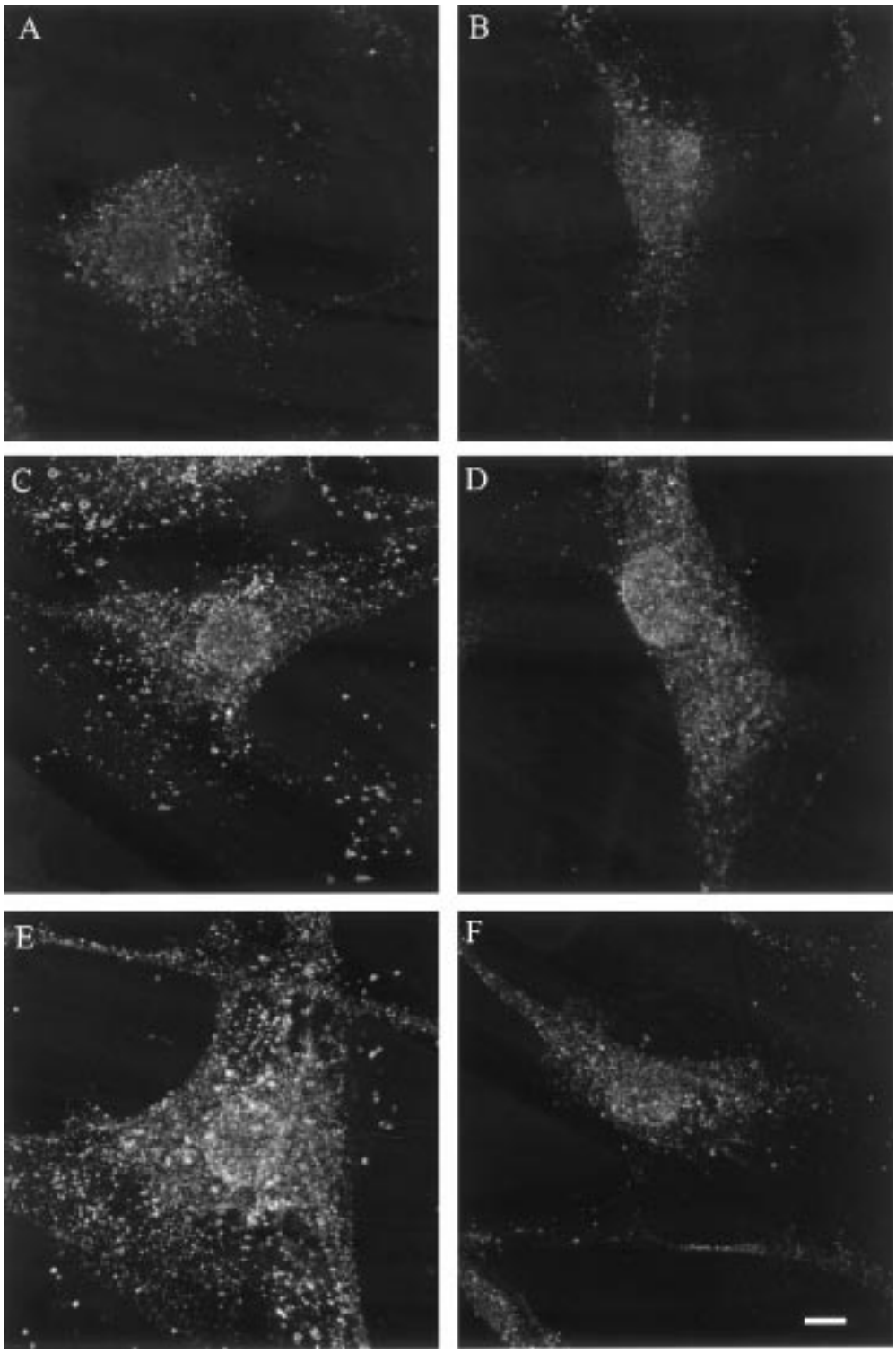

Fig. 7. Confocal immunofluorescence microscopic localization of PKCβII in orbital fibroblasts from patients with Graves' ophthalmology. Orbital fibroblasts seeded on coverslips were stained for immunofluorescence with anti-PKC βII antibody. **A**: Untreated orbital fibroblasts. **B**: Cells treated for 45 min with H-7 (20 μg/ml). **C**: Cells treated for 15 min with IL-1β (25 U/ml). **D**: Cells pretreated for 45 min with H-7 (20 μg/ml), then treated for 15 min with IL-1β (25 U/ml). **E**: Cells treated for 10 min with PMA (10 ng/ml). **F**: Cells pretreated for 45 min with H-7 (20 μg/ml), then treated for 10 min with PMA (10 ng/ml). In C and E, PKCβII was translocated to the cell membrane. In D and F, H-7 partially inhibited PKC βII translocation. Bar, 10 μm.

in both untreated and H-7 treated cells (Fig. 7A and B), and treatment with either IL-1β (Fig. 7C) or PMA (Fig. 7E) resulted in translocation of PKC βII to the membrane, this effect being abolished by H-7 (Fig. 7D and F).

DISCUSSION

Two signal transduction pathways, involving PKC [Klewes and Prehm, 1994; Suzuki et al., 1995] or cAMP dependent protein kinases [Honda et al., 1993; Klewes and Prehm, 1994; Imai et al., 1994; Suzuki et al., 1995], have been suggested to be involved in activating hyaluronan synthesis. Here, we show that both IL-1β and PMA can stimulate hyaluronan synthesis in orbital fibroblasts. Moreover, the effects of PMA and IL-1β were not additive (Fig. 3), suggesting an important role for PKC in the induction of hyaluronan synthesis in orbital fibroblasts. In contrast, forskolin, an adenyl cyclase and PKA activator, did not stimulate hyaluronan synthesis (Fig. 3). The involvement of PKC in IL-1β-induced hyaluronan synthesis in orbital fibroblasts was confirmed by blocking experiments using the PKC inhibitors, H-7 and chelerythrine chloride. In a related experiment, longer PMA treatment (12 h), which results in PKC down-regulation, was also shown to suppress hyaluronan synthesis (data not shown).

Neither PMA nor forskolin had an additive effect on IL-1β-induced hyaluronan synthesis. Interestingly, in the presence of the PKA activator, IL-1β- or PMA-induced hyaluronan synthesis was reduced (Fig. 3), possibly as a result of communication between the PKA and PKC pathways which may be relevant to the normal physiology of the orbit and the pathogenesis of inflammatory disease. The mechanism involved in the communication between these two kinases in hyaluronan synthesis is, however, not clear.

One possible mechanism for the IL-1β-induced activation of PKC is via activation of phospholipase C (PLC), which is known to interact with, and be phosphorylated by, the IL-1β receptor. PLC increases the levels of inositol 1, 4, 5-triphosphate and DAG, and this is followed by an increase in the $[Ca^{2+}]i$. A rise in the $[Ca^{2+}]i$ may facilitate the activation of key intracellular signaling molecules that lead to hyaluronan synthesis. cPKC is a one such Ca^{2+}-dependent signaling intermediary that can be activated by an increase in the $[Ca^{2+}]i$. Since Ca^{2+} can activate cPKCs and since PMA increased hyaluronan synthesis, we explored the potential role of cPKC in IL-1β-induced hyaluronan synthesis. As shown in Figure 4, the calcium chelator, BAPTA, blocked IL-1β-induced hyaluronan synthesis, and we therefore propose that IL-1β acts through a Ca^{2+}-sensitive PKC (a cPKC isoform) to induce hyaluronan synthesis in orbital fibroblasts. In our experiments, IL-1β or PMA treatment resulted in PKC activation, confirming the functionality of the enzyme in these cells (Table I).

The cellular signals responsible for Ca^{2+} mobilization and PKC activation have not been defined. Since calcium chelation was more effective than PKC inhibition in reducing IL-1β-induced hyaluronan synthesis, the effect of the $[Ca^{2+}]i$ on hyaluronan synthesis was investigated. A23187, a calcium ionophore that causes Ca^{2+} influx, induced hyaluronan synthesis (Fig. 5B). Our results suggest that an increase in the $[Ca^{2+}]i$ is necessary for receptor-mediated PKC translocation. However, little is known about the mechanism by which these factors elicit this response.

Moreover, IL-1β-stimulated hyaluronan production requires protein synthesis (Fig. 2), suggesting induction of expression of hyaluronan synthase mRNA and protein. This result is consistent with recent findings in orbital fibroblast cells that IL-1β can upregulate hyaluronan synthase mRNAs and that this effect can be blocked by cycloheximide [Kaback and Smith, 1999].

Translocation of PKC from the cytosol to membranes is used as an indicator of PKC activation. Following IL-1β or PMA stimulation, translocation of PKC βII to the membrane was seen (Fig. 7C, E), and the pattern of PKC βII immunoreactivity in IL-1β- or PMA-stimulated cells was affected by the PKC inhibitor, H-7 (Fig. 7D,E). In contrast, the immunohistochemical localization of PKCα and γ remained unchanged following PMA or IL-1β stimulation. These results suggest that the PKC βII isoform is involved in IL-1β-induced activation of the PKC pathway in orbital fibroblasts.

Orbital fibroblasts, together with macrophages and other immune cells, may contribute to the increase in cytokine levels. IL-1β, a proinflammatory cytokine, may contribute to the inflammatory events occurring in the orbit. Furthermore, PKC is involved in the

stimulation of hyaluronan synthesis in orbital fibroblasts and consequently plays an important role in the pathogenesis of Graves' ophthalmopathy. In summary, we have provided evidence that IL-1β induces hyaluronan synthesis and that cPKC is involved in this process. The effects of IL-1β and PMA on hyaluronan synthesis are dependent on protein synthesis and an increase in the $[Ca^{2+}]i$. Interruption of this PKC signaling pathway or reduction of the intracellular Ca^{2+} concentration may represent important therapeutic targets for Graves' ophthalmopathy. Information about the mechanism involved in the stimulation of hyaluronan synthesis would make it possible to modulate hyaluronan synthesis in clinical conditions characterized by hyaluronan overproduction.

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