PKC α Mediates CCL18-Stimulated Collagen Production in Pulmonary Fibroblasts

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A CC chemokine, CCL18, has been previously reported to stimulate collagen production in pulmonary fibroblasts. This study focused on the role of protein kinase C (PKC) in the profibrotic signaling activated by CCL18 in pulmonary fibroblasts. Of the three PKC isoforms that are predominantly expressed in fibroblasts (PKC α , PKC δ , and PKC ϵ), two isoforms (PKC δ and PKC ϵ) have been implicated in profibrotic intracellular signaling. The role of PKC α -mediated signaling in the regulation of collagen production remains unclear. In this study, PKC α was found mostly in the cytoplasm, whereas PKC δ and PKC ϵ were found mostly in the nucleus of cultured primary pulmonary fibroblasts. In response to stimulation with CCL18, PKC α but not PKC δ or PKC ϵ underwent rapid (within 5–10 min) transient phosphorylation and nuclear translocation. Inhibition with dominant-negative mutants of PKC α and ERK2, but not PKC δ or PKC ϵ , abrogated CCL18-stimulated ERK2 phosphorylation and collagen production. The effect of CCL18 on collagen production and the activity of collagen promoter reporter constructs were also abrogated by a selective pharmacologic inhibitor of PKC α Gö6976. Stimulation of fibroblasts with CCL18 caused an increase in intracellular calcium concentration. Consistent with the known calcium dependence of PKC α signaling, blocking of the calcium signaling with the intracellular calcium-chelating agent BAPTA led to abrogation of PKC α nuclear translocation, ERK2 phosphorylation, and collagen production. These observations suggest that in primary pulmonary fibroblasts, PKC α but not PKC δ or PKC ϵ mediate the profibrotic effect of CCL18. PKC α may therefore become a viable target for future antifibrotic therapies.

Keywords: chemokines; fibroblast; fibrosis; lung; signal transduction

Human CC chemokine ligand (CCL)-18, also called pulmonary and activation-regulated chemokine (PARC), macrophage inflammatory protein (MIP)-4, alternative macrophage activation-associated CC chemokine (AMAC)-1, dendritic cell chemokine (DC-CK1), and small secreted cytokine A (SCYA)-18, is produced at high levels in the lungs (1), particularly by alveolar macrophages that normally acquire alternatively activated phenotype (2). This chemokine is also produced, although to a lesser degree, by peripheral blood monocytes, tissue macrophages, and dendritic cells (3, 4). CCL18 attracts both naïve and activated CD4⁺ and CD8⁺ T cells, but not granulocytes or monocytes (1, 3).

Increased levels of CCL18 mRNA and/or protein have been reported in the lungs of patients with pulmonary fibrosis, such as in scleroderma lung disease (5), hypersensitivity pneumonitis and idiopathic pulmonary fibrosis (6), pulmonary sarcoidosis (7), and in a primate model of allergic asthma (8). We recently

reported that CCL18 acts directly on human and mouse pulmonary fibroblasts and stimulates collagen production in a time- and dose-dependent fashion (9, 10). CCL18 and CCL2 (MCP-1) are the only known chemokines with direct profibrotic effects *in vitro*, yet the mechanisms of action of these two CC chemokines on pulmonary fibroblasts are likely different (10, 11). Predominant expression in the lung tissue, association of increased levels with pulmonary fibrosis, and direct profibrotic activity on pulmonary fibroblasts make CCL18 a potential target of pulmonary antifibrotic therapies.

Lung scarring is a major cause of death in scleroderma lung disease, idiopathic pulmonary fibrosis, radiation- and chemotherapy-induced lung fibrosis, rheumatoid arthritis, graft-versus-host disease after bone marrow transplantation, and conditions caused by occupational inhalation of dust particles (reviewed in Ref. 12). Protein kinase C (PKC)-mediated signaling regulates fibroblast activities leading to fibrosis, particularly fibroblast proliferation and collagen production. Fibroblasts are known to express three PKC isoforms: PKC α , PKC δ , and PKC ϵ (13, 14). Previous reports focused mainly on the roles of PKC δ and PKC ϵ in fibrosis. TGF- β regulates collagen synthesis in pulmonary fibroblasts not only through the Smad pathway, but also through PKC δ ; conversely, IL-7 inhibits the profibrotic effect of TGF- β on pulmonary fibroblasts by not only inhibiting Smads, but also by inhibiting the PKC δ activity (15). In pulmonary fibroblasts, TGF- β induces PKC-dependent signaling through the Raf-MEK-MAPK signaling pathway (16). Production of collagen type I and type III is PKC δ -dependent in dermal fibroblasts from patients with systemic sclerosis (scleroderma) and from normal healthy control subjects (17). Pharmacologic and dominant-negative mutant-mediated inhibition of PKC δ in dermal fibroblasts decreased collagen gene expression through Sp1/Sp3- and Ets-dependent signaling (18). Thrombin-induced myofibroblast transformation (19), resistance to apoptosis (20), and tenascin (21) and collagen (22) expression in pulmonary fibroblasts are PKC ϵ -dependent.

In contrast to the wealth of information on PKC δ and PKC ϵ , very limited data are available on the role of PKC α -mediated signaling in the regulation of collagen production. One recent report (18) suggested that inhibition of PKC δ and PKC α signaling downregulates basal collagen production in dermal fibroblasts. The only other recent report (22) suggested that PKC α is involved in a complex PKC-caveolin-MEK-ERK loop, activity of which regulates basal collagen production. Involvement of PKC α in a cytokine-mediated regulation of collagen production has not been investigated. It is not known whether PKC is involved in the CCL18-stimulated profibrotic signaling.

Delineation of intracellular signaling pathways activated by CCL18 in pulmonary fibroblasts is important for development of future antifibrotic therapies. In this article we show for the first time that CCL18 activates PKC α but not PKC δ or PKC ϵ phosphorylation and nuclear translocation in primary pulmonary fibroblasts. Using pharmacologic inhibitors and overexpression of dominant-negative constructs, we show that CCL18-stimulated ERK2 phosphorylation, the activity of the collagen promoter constructs, and ultimately collagen production are likely

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PKC α -dependent but PKC δ - and PKC ϵ -independent. We also report for the first time that CCL18 induces calcium flux that is critical for the PKC α activation and the PKC α -dependent events. We conclude that PKC α signaling is profibrotic in pulmonary fibroblasts and that it is central to the stimulating effect of CCL18 on collagen production.

MATERIALS AND METHODS

Primary Pulmonary Fibroblast Cultures

Three normal human primary pulmonary fibroblast cultures (NLF1–NLF3), all from different donors, were used in this study. The normal adult fibroblast cultures NLF1 and NLF2 were purchased from Cambrex (Walkersville, MD) and normal fetal fibroblast culture NLF3 (IMR-90) from American Type Culture Collection (Manassas, VA). Fibroblast cultures were maintained in T75 culture flasks in humidified atmosphere of 5% CO₂ at 37°C in high serum tissue culture medium, which was DMEM supplemented with 2 mM glutamine, 2 mM sodium pyruvate, 50 mg/liter gentamicin, and 10% bovine calf serum (all from Invitrogen, Carlsbad, CA). Cell cultures used in experiments were preincubated for 24 h in similar conditions, using low-serum (0.5% dialyzed bovine calf serum with no TGF- β detectable by ELISA) medium supplemented with 0.28 mM ascorbic acid and 0.2 mM β -aminopropionitrile (Sigma, St. Louis, MO) in addition to the mentioned reagents. The cell culture medium for all experiments was the same low-serum medium. In all experiments, fibroblast cell lines were tested in passages three to seven.

Materials

Recombinant human (rh) CCL18 was purchased from R&D Systems (Minneapolis, MN) and used to stimulate collagen production in fibroblast cultures at 300 ng/ml unless specified otherwise (9, 10). TGF- β 1 (R&D Systems) was used as a positive control for fibroblast stimulation at 1 ng/ml. Selective PKC α /PKC β 1 inhibitor Gö6976 was purchased from Calbiochem (La Jolla, CA) and was 98% chromatographically pure and quality control tested by the supplier. There is little, if any, expression of PKC β 1 in fibroblasts (23), thus making Gö6976 selective for PKC α in these cells. Cell viability in the presence of the inhibitor was determined using Trypan Blue exclusion assays.

Well-characterized plasmid construct for expression of dominant-negative mutants (DNM) were generous gifts from Dr. Matthew Young (ERK2 DNM; *see* Ref. 24) and Dr. Jae-Won Soh (PKC α , PKC δ , and PKC ϵ DNMs, and pcDNA3 plasmid as a negative control; *see* Ref. 25). Collagen promoter–chloramphenicol acetyltransferase (CAT) reporter constructs were a kind gift from Dr. Maria Trojanowska (Medical University of South Carolina, Charleston, South Carolina). The library of constructs included fragments of human COL1A2 promoter (–108, –186, –353, –772, and –3,500 basepairs relative to the transcription start site; 26) cloned in the proper orientation upstream from the chloramphenicol acetyltransferase reporter gene. Production of chloramphenicol acetyltransferase was measured in CAT ELISA assays (Roche, Indianapolis, IN) according to manufacturer's recommendations.

Antibodies against phosphorylated and total PKC α and PKC δ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated and total PKC ϵ were purchased from Upstate (Lake Placid, NY). Antibodies against phosphorylated and total ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA), and antibodies against phosphorylated and total ERK2 were purchased from Upstate.

Immunofluorescence Analyses

Fibroblasts were cultured on chamber slides and activated with rhCCL18 for 5, 10, 15, 30, and 60 min. The cells then were fixed with 4% paraformaldehyde, permeabilized with 0.05% Tween 20 in PBS, and blocked with 5% BSA in 0.05% Tween 20 in PBS. Then, cells were incubated with primary rabbit antibodies against human PKC isoforms and reacted with FITC-conjugated anti-rabbit IgG (Upstate). To visualize the nuclei, the cells were stained with DAPI or propidium iodide. Fluorescent images were observed and digitally acquired using Axiovert 200 fluorescent microscope (Carl Zeiss, Jena, Germany) at $\times 400$ magnification. Green (for PKC staining) and red or blue (propidium iodide

or DAPI, respectively) fluorescence channels were captured separately and overlaid digitally.

Collagen Production Assays

Production of collagen in 24–48 h fibroblast cultures was measured by metabolic incorporation of ¹⁴C-proline into collagenase-sensitive protein. Briefly, cultured fibroblasts were pulsed with 1 μ Ci/ml of ¹⁴C-proline for the final 6 h, and the cells were lysed with three freezing-thawing cycles. Then, each sample was divided into two equal parts, and one part digested with 50 U/ml collagenase III (Calbiochem, San Diego, CA), followed by precipitation of both parts with 10% trichloroacetic acid (TCA) in 0.1% L-proline. After three washings with 5% TCA and two washings with ice-cold 95% ethanol, the collagen production was expressed as the collagenase-susceptible incorporated radioactivity in CPM.

Alternatively, collagen type I was quantified in 48 h fibroblast cultures as described elsewhere (9, 10). Briefly, fibroblasts were cultured with β -aminopropionitrile (Sigma) to prevent collagen cross-linking, and Western blotting assays for collagen were performed using rabbit affinity purified anti-collagen type I antibody (Rockland, Gilbertsville, PA). Before electrophoresis, samples were reduced and denatured by boiling in Laemmli buffer containing β -mercaptoethanol. Human purified collagen type I (Southern Biotech, Birmingham, AL) was used as a positive control in these assays. The identity of collagen bands was confirmed by sensitivity to pepsin and collagenase (both from Sigma) digestion, as described (9, 10). Images were collected using a Storm densitometer, and band densities were analyzed after adjustment to the local background with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Selected results were confirmed using metabolic labeling of collagen with ¹⁴C-proline, followed by electrophoretic separation with subsequent fluorographically enhanced autoradiography, as previously described (9, 10).

Immunoblotting for Phosphorylated and Total PKC Isoforms and ERK1/2

Fibroblasts were plated in 6-well plates (Costar, Cambridge, MA) at 2×10^5 cells/well in 3-ml cultures. After incubation with rhCCL18 for various periods of time, fibroblast cultures were washed with ice-cold PBS and lysed with 250 μ l of Laemmli sample buffer. To obtain nuclear lysates, the Nuclear Extract Kit (Active Motif, Carlsbad, CA), was used following the manufacturer's instructions. Electrophoretic separation of the lysates was done in polyacrylamide gels, and bands were transferred onto Immobilon NC membranes (Millipore, Bedford, MA). Membranes were probed with specific primary antibodies at 1/200 dilution, followed by secondary HRP-conjugated antibodies (Upstate), and visualized with an ECL detection system (Pierce, Rockford, IL) that was used according to the manufacturer's directions. Bands for the total isoform proteins were developed with antibodies for total PKC isoforms or ERK1/2 after stripping the membranes developed with anti-phospho-PKC or anti-phospho-ERK1/2 antibodies, respectively. Gel images were collected using a Storm densitometer and band densities analyzed with ImageQuant software (Molecular Dynamics).

Transient Transfection of Primary Fibroblast Cultures

Transfections were performed using Metafectene (Biontex, Munich, Germany) and Mirus (Madison, WI) transfection reagents, using 2.5 μ g of plasmid per well in 6-well plates. Cultures were then washed with fresh medium, rested for 24 h, and tested in subsequent assays. Thirty-five to forty percent of fibroblasts expressed GFP by fluorescent microscopy 24 h after transfection or co-transfection with pEGFP-C1 vector (BD Biosciences Clontech, Palo Alto, CA), and further electronic accumulation of the fluorescent signal revealed that 60–80% of cells had higher levels of fluorescence than the control mock-transfected cells. Also, Western blotting analyses were used to directly confirm the relative overexpression of dominant-negative mutants for the targeted PKC isoforms and ERK1/2.

Measurement of Cytosolic Free Ca²⁺ Concentration

Fibroblasts were plated on 25-mm coverslips and were incubated in culture medium containing 3.3 μ M fura 2-AM (Molecular Probes, Eugene, OR) for 30 min at 37°C in the humidified atmosphere of 5%

CO₂ in air. The fura 2-AM-loaded cells were then superfused with standard bath solution for 20–30 min at 22–24°C to wash away extracellular dye and permit intracellular esterases to cleave cytosolic fura 2-AM into active fura 2. Fura 2 fluorescence from the cells and background fluorescence were imaged using a Nikon Diaphot microscope (Nikon, Melville, NY) equipped for epifluorescence. Fluorescent images were obtained using a microchannel plate image intensifier (Amperex XX1381; Opelco, Washington, DC) coupled by fiber optics to a Pulnix charge coupled device video camera (Stanford Photonics, Stanford, CA). Image acquisition and analysis were performed with a Metamorph Imaging System (Universal Imaging, Downingtown, PA). The ratio imaging of cytosolic free Ca²⁺ concentration [Ca²⁺]_{cyt} was obtained from fura 2 fluorescent emission excited at 380 and 340 nm. An intracellular calcium chelator BAPTA AM was purchased from Molecular Probes and used in final concentration 10–50 μM.

Statistical Analyses

Data were expressed as mean value ± SD. Differences between two groups were evaluated with a two-tailed unequal variance Student's *t* test. Differences among more than two groups were evaluated by one-way ANOVA with *post hoc* (Scheffe) testing. A *P* value < 0.05 was considered statistically significant. Statistical analyses were performed using Statistica software (StatSoft, Tulsa, OK).

RESULTS

Effect of CCL18 on Collagen Production

Considering the well-known phenotypic heterogeneity of primary fibroblasts (27), the initial experiments were performed to determine whether the fibroblast cultures in this work respond to stimulation with rhCCL18 by accelerating collagen production. ¹⁴C-Proline incorporation assays revealed that exposure to rhCCL18 accelerated production of collagenase-sensitive protein in cultured fibroblasts in a dose-dependent fashion, as shown in Figure 1. The effect of CCL18 on collagen production was observed at doses 100 ng/ml and above in all tested cultures. Further analyses of collagen type I production in response to 300 ng/ml rhCCL18 were performed by using this technique or by Western blotting, as shown below.

Immunofluorescent Analysis of PKC Isoform Expression in Pulmonary Fibroblasts

The immunostaining assays for the PKC isoforms normally found in fibroblasts (13, 14) were performed to assess their ex-

Figure 1. Effect of rhCCL18 on collagen synthesis. Fibroblast cultures were treated for 24 h with indicated doses of rhCCL18. The incorporation of ¹⁴C-proline into collagenase-sensitive protein was expressed as fold CPM increase in CCL18-stimulated cultures versus untreated controls, mean ± SD. Stimulation was statistically significant where indicated with asterisks. Data from a representative experiment in NLF1 are shown. NLF2 and NLF3 showed similar responses to CCL18 stimulation (not shown). Triplicate cultures were tested in all cases in at least two independent experiments.

pression and subcellular localization in the studied fibroblast cultures. Intracellular staining for PKC isoforms revealed that PKCα, PKCδ, and PKCε were all present in pulmonary fibroblasts (Figure 2). In all cases, PKCα was located in the cytoplasm but not in the nucleus of fibroblasts (Figures 2A and 2B); expression of PKCδ was intranuclear and, to a lesser extent, cytoplasmic (Figures 2C and 2D), whereas PKCε was almost exclusively intranuclear (Figures 2E and 2F).

Dynamics of CCL18-Stimulated PKC Phosphorylation

Subsequent experiments addressed the question whether stimulation of fibroblasts with rhCCL18 activates PKC isoforms. Western blotting analyses revealed that PKCα was basally phosphorylated in pulmonary fibroblasts and underwent a rapid transient increase in phosphorylation after fibroblast exposure to CCL18 (Figure 3). The PKCα phosphorylation kinetics were similar in nontransfected, mock-transfected, transfected with PKCδ DNM or PKCε DNM, and transfected with the control plasmid fibroblast cultures (only the latter representative result is shown in Figures 3A and 3B). In the fibroblasts cultures overexpressing the PKCα dominant-negative mutant construct, basal phosphorylation was preserved, but the CCL18-stimulated increase in phosphorylation was abrogated (Figures 3C and 3D). There was no similar increase in PKCδ or PKCε phosphorylation above the basal level after fibroblast stimulation with CCL18 (Figures 3E–3H); various concentrations of CCL18 up to 1,000 ng/ml were also tested and had no effect on PKCδ or PKCε phosphorylation (data not shown). Immunohistochemical evaluations and Western blotting analyses of nuclear lysates revealed that PKCα content in the nuclei increases transiently at 5–10 min after stimulation with CCL18 (Figure 4). These observations suggested that although the three PKC isoforms were present, only PKCα but not PKCδ or PKCε became phosphorylated as part of the CCL18-stimulated signaling in pulmonary fibroblasts. PKCα also underwent transient nuclear translocation in response to stimulation with CCL18. Together, these observations suggest that PKCα but not PKCδ or PKCε is activated by CCL18.

PKCα Dependence of CCL18-Stimulated ERK2 Phosphorylation and Collagen Production

Further Western blotting analyses were performed to determine whether PKC signaling effects ERK signaling. Exposure of fibroblasts to CCL18 caused an upward mobility shift of the p42 band and a transient increase in ERK2 phosphorylation, effects that were significantly reduced by the overexpression of the PKCα dominant-negative mutant construct (Figure 5A). Overexpression of PKCδ or PKCε dominant-negative mutant constructs had no effect on CCL18-stimulated ERK2 phosphorylation (data not shown). These observations suggested that CCL18-stimulated ERK2 phosphorylation is PKCα-dependent but PKCδ- and PKCε-independent. Overexpression of the dominant-negative ERK2 inhibited basal collagen production and CCL18-stimulated collagen upregulation as well as basal and CCL18-stimulated ERK2 phosphorylation (Figure 5B). Pharmacologic inhibitor of PKCα Gö6976 abrogated CCL18-stimulated but not TGF-β1-stimulated collagen upregulation at 500 nM but not 100 nM concentration (Figures 5C and 5D). Finally, overexpression of PKCα DMN construct abrogated CCL18-stimulated collagen upregulation, whereas overexpression of PKCδ or PKCε DNM constructs had no effect on CCL18-stimulated collagen upregulation (Figure 5E). These observations further argued against PKCδ or PKCε involvement in CCL18-stimulated signaling and suggested that CCL18-stimulated ERK2 phosphorylation is PKCα-dependent and that CCL18-stimulated collagen upregulation is both ERK2-dependent and PKCα-dependent.

Figure 2. Immunofluorescent staining of pulmonary fibroblasts (NLF1) for PKC α (A), PKC δ (C), and PKC ϵ (E) alone and overlaid with the corresponding propidium iodide (B, F) or DAPI (D) nuclear staining. The location of PKC α was mainly cytoplasmic (A, B), whereas PKC δ (C, D) and PKC ϵ (E, F) were located mostly in the nucleus. The staining was performed in fibroblast derived from all donors in this study (NLF1–NLF3), on at least four occasions in each case, at various passages, with consistent results. No staining was detected with isotype control primary antibodies (not shown).

PKC α -Dependent Responsiveness of the Collagen Promoter to CCL18 Stimulation

Experiments were performed to determine whether PKC α signaling mediates the transcriptional regulation of CCL18-dependent collagen production. Primary pulmonary fibroblast cultures were transfected with a series of COL1A2 promoter deletion–chloramphenicol acetyltransferase reporter constructs. As shown in Figure 6, the –3,500 bp and –772 bp constructs responded to

Figure 3. Western blotting of fibroblast lysates for indicated PKC isoforms after stimulation of fibroblast cell cultures with rhCCL18 for indicated times. Fibroblast cultures were transfected with control pcDNA3 plasmid (A, B), the plasmid encoding PKC α DNM (C, D), or not transfected in all other cases (E–H). PKC α but not PKC δ or PKC ϵ underwent rapid transient phosphorylation at 5 and 10 min after activation with CCL18 (tested with consistent results in at least three different experiments in each of the three studied primary fibroblast cultures). The densities of phospho-PKC α bands related to the densities of corresponding total PKC α bands were increased 4.5- $\pm$ 1.2-fold at 5 min and 2.9- $\pm$ 0.7-fold at 10 min versus the densities at other times (P < 0.05, one-way ANOVA); there was no increase at other times (P > 0.05).

Figure 4. Immunofluorescent staining of permeabilized pulmonary fibroblasts (NLF3) for PKC α before (A) and 5 min after stimulation with rhCCL18 (B). Nuclei (indicated with arrows) stain more intensively at 5 min after stimulation (B) than before stimulation (A) or 30 min after stimulation (not shown) with rhCCL18. There was no change in the subcellular distribution patterns of PKC δ and PKC ϵ after stimulation with rhCCL18 (not shown); both isoforms remained predominantly intranuclear (see Figures 2C–2F). (C) Western blotting of nuclear lysates revealed that nuclear PKC α content rapidly and transiently increased at 5 min and declined to basal level at 15 min after stimulation (nuclear lysates were normalized for total protein, repeated twice in each NLF1 and NLF3 with consistent results).

Figure 5. Western blotting for ERK phosphorylation at indicated times and collagen after 48 h of fibroblast stimulation with rhCCL18. (A) Overexpression of PKC α but not transfection with the control plasmid abrogates CCL18-stimulated ERK2 phosphorylation. Overexpression of PKC δ or PKC ϵ DNM did not inhibit ERK2 phosphorylation in response to CCL18 (not shown). These experiments were repeated on three different occasions in each NLF1 and NLF3, with consistent results. (B) Overexpression of ERK2 DNM abrogated basal and CCL18-stimulated collagen production (48 h after stimulation), and basal and CCL18-stimulated ERK2 phosphorylation (15 min after stimulation, repeated on two different occasions in NLF1 and NLF3, with consistent results). (C) Pharmacologic inhibitor of PKC α Gö6976 abrogated CCL18-stimulated collagen upregulation at 500 nM but not 100 nM concentration (repeated on two different occasions in each of the tested primary fibroblast cultures, with similar results). (D) Pharmacologic inhibitor of PKC α Gö6976 abrogated CCL18-stimulated (filled bars) but not TGF- β 1-stimulated (open bars) collagen production in NLF3 (14 C-proline incorporation assay, significant differences from nonstimulated controls indicated with asterisks, $P < 0.05$). (E) Overexpression of PKC α , unlike mock transfection or overexpression of PKC δ or PKC ϵ , abrogated CCL18-stimulated collagen. Lanes labeled with "c" and "s" correspond to control and CCL18-stimulated cell culture supernatants, respectively (repeated three times in NLF3, with consistent results).

the CCL18 stimulation, whereas the -353 bp construct responded with a significantly smaller amplitude and the -186 bp and -108 bp constructs showed no response. These observations suggest the location of CCL18-responsive elements in the collagen promoter is located between -772 bp and -186 bp, although the detailed identification of these elements was outside the scope of this work. Importantly, CCL18 responsiveness was inhibited in the presence of Gö6976 (Figure 6), indicating that the transcriptional regulation of the collagen promoter is PKC α -dependent.

Calcium Dependence of PKC α -Mediated CCL18 Signaling

To further confirm that PKC α but not PKC δ or PKC ϵ is involved in CCL18 signaling in pulmonary fibroblasts, we relied on calcium dependence of PKC α -mediated signaling and calcium independence of PKC δ - and PKC ϵ -mediated signaling. Stimulation of pulmonary fibroblast cultures with CCL18 caused an increase in $[Ca^{2+}]_{cyt}$ (Figures 7A and 7C) that was inhibited by the presence of a calcium chelator BAPTA (Figures 7B and 7C). Chelation of calcium with BAPTA also abrogated PKC α nuclear translocation, ERK2 phosphorylation, and upregulation in collagen

production (Figures 7D and 7E). These observations suggested that CCL18-stimulated signaling is calcium-dependent, thus further arguing in support of PKC α but not PKC δ or PKC ϵ involvement.

DISCUSSION

Levels of CCL18 mRNA and protein are increased in the lungs of patients with pulmonary fibrotic diseases (5–8). CCL18 may contribute to pulmonary fibrosis by selectively attracting profibrotic T cells to the lung (28–30). In addition, CCL18 acts directly on cultured primary adult pulmonary fibroblasts and upregulates collagen production (9, 10). There is only one other CC chemokine, CCL2, known to activate collagen production in pulmonary fibroblasts, but its action is likely to be mediated by autocrine TGF- β 1 (11). In contrast, CCL18 is a likely transcriptional activator of collagen production acting through a G protein-dependent, ERK1/2-dependent, p38-independent pathway, and Sp1-dependent pathway that does not require autocrine TGF- β 1 but requires basal activity of Smad3 (9, 10). These observations together suggested that CCL18 may be an attractive therapeutic target in pulmonary fibrosis.

Figure 6. Responsiveness of the deletion COL1A2 promoter-CAT reporter constructs to CCL18 without (filled bars) or with (open bars) 500 nM Gö6976. Stimulation with CCL18 led to significant activation of CAT production in the fibroblast cultures transfected with the constructs indicated with single asterisks ($P < 0.05$). Inhibition with Gö6976 significantly abrogated this response where indicated with double asterisks ($P < 0.05$). Mean $\pm$ SD fold induction versus nonactivated cultures transfected with the corresponding constructs from four to five independent experiments are presented. Relative positions of some of the known transcription factor binding sites in COL1A2 promoter are shown on the left (not to scale).

The role of PKC δ and PKC ϵ in profibrotic regulation of fibroblasts has been convincingly shown before (15–22), whereas the available data on PKC α remain limited. The goal of our present study was to investigate the early signaling events activated by CCL18 in pulmonary fibroblasts, particularly involvement of PKC isoforms. The studied primary adult pulmonary fibroblast cultures responded to stimulation with rhCCL18 by increasing collagen production (see Figures 1, 5, and 7) in a fashion similar to that of CCL2 (9, 11). Pulmonary fibroblasts expressed PKC isoforms α , δ , and ϵ , with PKC α located in the cytoplasm and PKC δ and PKC ϵ located predominantly in the nucleus (see Figures 2 and 4). Together, these observations suggested that the well-known phenotypic heterogeneity of primary fibroblasts (27) does not involve the responsiveness to stimulation with CCL18 or PKC expression pattern in the primary fibroblast cultures used in this study. The observed subcellular localization of the PKC isoforms (see Figure 2) also suggested that the predominantly cytoplasmically localized PKC α has the potential for nuclear translocation upon activation, whereas PKC δ and PKC ϵ are already localized mostly in the nucleus. We observed that PKC α but not PKC δ or PKC ϵ undergoes rapid transient phosphorylation (see Figure 3) and nuclear translocation (see Figure 4) in response to fibroblast stimulation with CCL18. We overexpressed the well-characterized DMNs to selectively target PKC isoforms and ERK2 (24, 25); the overexpression was confirmed by Western blotting (an example is shown in Figure 5B, bottom gel). Overexpression of PKC α DNMs but not PKC δ or PKC ϵ DNMs abrogated CCL18-stimulated PKC α phosphorylation (see Figure 3), and ERK2 phosphorylation (see Figure 5A). Interestingly, ERK2 (p42) but not ERK1 (p44) is the form that is predominantly activated by CCL18 (see Figures 5A and 5B). The CCL18-stimulated collagen upregulation was ERK2-dependent (see Figure 5B) and was also inhibited by the overexpression of PKC α DNMs but not PKC δ or PKC ϵ DNMs,

and by the PKC α pharmacologic inhibitor Gö6976 (another target of this inhibitor, PKC β 1, is not expressed in fibroblasts; 23). Based on these observations, we concluded that the CCL18-stimulated ERK2-dependent collagen upregulation is PKC α -dependent but not PKC δ - or PKC ϵ -dependent. We employed a battery of collagen promoter–chloramphenicol acetyltransferase reporter constructs to determine whether the involvement PKC α in the regulation of CCL18 effect extends down to the collagen promoter level. The PKC α inhibitor Gö6976 abrogated the responsiveness of the –3,500, –772, and –353 collagen promoter reporter constructs, suggesting that the entire CCL18-stimulated transcriptional regulation of collagen expression is PKC α -dependent (see Figure 6). We report for the first time that CCL18 induces calcium flux in pulmonary fibroblasts that can be inhibited by the calcium chelator BAPTA (see Figures 7A–7C). Interestingly, BAPTA also inhibited CCL18-stimulated PKC α nuclear translocation, ERK2 phosphorylation, and collagen upregulation (see Figures 7D and 7E). Considering the known calcium dependence of PKC α -mediated but not PKC δ - or PKC ϵ -mediated signaling, these observations further support the central role of PKC α but not PKC δ or PKC ϵ in the CCL18-stimulated profibrotic signaling in pulmonary fibroblasts.

In conclusion, PKC α , but not PKC δ or PKC ϵ , is central to CCL18-stimulated intracellular signaling and upregulation of collagen production in normal pulmonary fibroblasts. In combination with our previous observations (5, 9, 10), this report suggests that CCL18 is not only a chemotactic factor for T cells, but also a direct regulator of collagen production in the lung, that acts by activating the Ca⁺⁺-dependent, PKC α -dependent, and ERK2-dependent pathway, leading to the transcriptional activation of the collagen promoter. This pathway may become an important target for future antifibrotic therapies in CCL18-mediated pulmonary fibrotic diseases.

Figure 7. Calcium-dependence of CCL18-induced signaling in pulmonary fibroblasts. (A and B) CCL18 induced Ca^{2+} influx in lung fibroblasts. A representative record showing time course of $[Ca^{2+}]_{cyt}$ changes induced by 300 ng/ml CCL18 in the presence of extracellular Ca^{2+} (1.8 mM; A), and in the presence of CCL18 and 50 μ M BAPTA (B). (C) Summarized data showing basal $[Ca^{2+}]_{cyt}$ and the amplitudes of CCL18-induced Ca^{2+} influx as shown in A. Values are mean $\pm$ SE ($n = 25$), * $P < 0.05$ versus control. (D) Western blotting of nuclear lysates indicated that BAPTA abrogated nuclear translocation of PKC α at 5 min after stimulation with CCL18 (top gel). Western blotting of whole fibroblast lysates indicated that BAPTA also abrogated ERK2 phosphorylation at 15 min after stimulation with CCL18 (middle gel), whereas total ERK content remained constant (bottom gel). Representative gels from two independent experiments in each NLF1 and NLF3 are shown. (E) The CCL18-stimulated collagen upregulation is inhibited by BAPTA in NLF1. The increase versus nonstimulated control was significant ($P < 0.05$) where indicated with the asterisk.

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