Protein kinase Cζ (PKCζ) is an intracellular serine/threonine protein kinase that has been implicated in the signaling pathways for certain inflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α), in some cell types. A study of gene expression in articular chondrocytes from osteoarthritis (OA) patients revealed that PKCζ is transcriptionally up-regulated in human OA articular cartilage clinical samples. This finding led to the hypothesis that PKCζ may be an important signaling component of cytokine-mediated cartilage matrix destruction in articular chondrocytes, believed to be an underlying factor in the pathophysiology of OA. IL-1 treatment of chondrocytes in culture resulted in rapidly increased phosphorylation of PKCζ, implicating PKCζ activation in the signaling pathway. Chondrocyte cell-based assays were used to evaluate the contribution of PKCζ activity in NF-κB activation and extracellular matrix degradation mediated by IL-1, TNF, or sphingomyelinase. In primary chondrocytes, IL-1 and TNF-α caused an increase in NF-κB activity resulting in induction of aggrecanase-1 and aggrecanase-2 expression, with consequent increased proteoglycan degradation. This effect was blocked by the pan-specific PKC inhibitors RO 31-8220 and bisindolylmaleimide I, partially blocked by Gö 6976, and was unaffected by the PKCζ-sparing inhibitor calphostin C. A cell-permeable PKCζ pseudosubstrate peptide inhibitor was capable of blocking TNF- and IL-1-mediated NF-κB activation and proteoglycan degradation in chondrocyte pellet cultures. In addition, overexpression of a dominant negative PKCζ protein effectively prevented cytokine-mediated NF-κB activation in primary chondrocytes. These data implicate PKCζ as a necessary component of the IL-1 and TNF signaling pathways in chondrocytes that result in catabolic destruction of extracellular matrix proteins in osteoarthritic cartilage.

Osteoarthritis (OA), a progressive and ultimately debilitating orthopedic disorder, is the most common degenerative joint disease in man (1). More than 20 million individuals in the United States alone have symptomatic OA, and it has been estimated that many more, greater than 50% of people over 65 years of age and 80% of those over age 75, have radiographic evidence of this disease (2). Yet, despite the widespread incidence of the disease in the human population, the etiology of OA is still largely unknown. OA is characterized by a slow focal destruction of articular cartilage, causing a roughening and thinning of the weight-bearing regions of the articular surface resulting in progressive immobility and pain. Articular cartilage is the tissue that provides shock-absorptive resiliency as well as low friction articulation to joints. It contains only a single cell type, the chondrocyte, which is responsible for the homeostasis of the tissue by synthesizing extracellular matrix that surrounds the cells and provides the important biophysical characteristics of the tissue. However, chondrocytes are also capable of producing catabolic factors capable of destroying the matrix components. Thus, extracellular matrix synthesis and degradation are dynamic processes that must be balanced by the chondrocytes for proper homeostasis of the tissue. In osteoarthritic cartilage, this balance appears to be shifted toward degradation, resulting in progressive loss of matrix due to up-regulation of proteolytic activities such as matrix metalloproteinases and aggrecanases. There is some debate whether osteoarthritis is a non-inflammatory arthropathy or an inflammatory arthritis; however, synovial inflammation has been documented in OA (3) and inflammatory cytokines, especially interleukin-1 (IL-1) and tumor necrosis factor (TNF), have been implicated as important mediators of the disease (4–8). These proinflammatory cytokines are known to be major regulators of chondrocytic expression of downstream proteases (such as aggrecanases and collagenases) that are ultimately involved in matrix breakdown resulting in the formation of osteoarthritic lesions (6, 8). Therefore, these cytokines themselves, or components of their intracellular signaling pathways, constitute possible therapeutic approaches for the treatment of OA.

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2 The abbreviations used are: OA, osteoarthritis; PKCζ, protein kinase Cζ; IL-1, interleukin-1; TNFα, tumor necrosis factor α; nPKC, novel PKC; aPKC, atypical PKC; RT, reverse transcriptase; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco’s modified Eagle’s medium; cPKC, conventional PKC; MES, 4-morpholineethanesulfonic acid; PS, pseudosubstrate; GO6976, sphingomyelinase inhibitor; Bpa, bisindolylmaleimide I; FBS, fetal bovine serum; DMMB, dimethylmethylene blue; m.o.i., multiplicity of infection; S5a, sphingomyelinase; Bts, bisindolylmaleimide I; KO, knock-out; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; MES, 4-morpholineethanesulfonic acid; P5, pseudosubstrate; CalC, calphostin C; CMV, cytomegalovirus.
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EXPERIMENTAL PROCEDURES

Media, Chemicals, and Reagents—Sphingomyelinase, collagenase, Pronase, penicillin/streptomycin, and RO 31–8220 (a broad spectrum PKC inhibitor also capable of activating JNK-1 (15)) were from Sigma. Recombinant human IL-1α, IL-1β, and TNF-α were purchased from R & D Systems. The following inhibitors were purchased from Calbiochem: bisindolylmaleimide I (a staurosporin analog that is a pan-PKC inhibitor (16)); calphostin C (a PKC inhibitor that does not inhibit atypical PKCs (17, 18)); and the NF-κB cell-permeable peptide inhibitors SN50 and the mutated control peptide SN50M (19). SN50 contains the nuclear localization sequence (residues 360–369) of the transcription factor NF-κB p50 subunit linked to the hydrophobic region (h-region) of the signal peptide of Kaposi fibroblast growth factor; SN50M is identical except this control peptide has two amino acid changes (Lys-363 to Asn and Arg-364 to Gly) that abolish its inhibition of NF-κB nuclear translocation. Triptolide (an NF-κB inhibitor compound (20)), G6 6976 (a PKC inhibitor with higher potency for conventional PKC family members (21)), and pseudosubstrate peptides for PKCζ (myristoylated and nonmyristoylated N-Ser-Ile-Tyr-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu) and PKCα/β (N-Myr-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-NH2) were purchased from Biomol (Plymouth Meeting, PA). Adenovirus expressing an NF-κB-luciferase reporter gene construct (22) was obtained from Dr. Doug Harnish (Wyeth Research). T/C-28a2 cells (23) were a generous gift from Dr. Mary Goldring.

Primary Chondrocyte Isolation and Culture—Bovine cartilage was obtained from the metacarpophalangeal joint of calves (2–10 days old), and chondrocytes were isolated by serial enzymatic digestion using Pronase (1 mg/ml, 37 °C for 30 min) and collagenase (1 mg/ml, 37 °C for overnight) in Dulbecco’s modified Eagle’s medium (DMEM) with 10 mM HEPES, and 100 units/ml penicillin, 100 μg/ml streptomycin. The digest was filtered through a 70-μm nylon cell strainer (Falcon) and processed as described (24). Cells were resuspended in growth media (HL-1 media, Cambrex catalog number 77201) containing 2 μM l-glutamine, 50 μg/ml ascorbate, and 10% fetal bovine serum (FBS) and aliquoted into 15-ml Falcon centrifuge tubes at a concentration of 1 × 106 cells per tube. The tubes were centrifuged at 200 × g at room temperature to allow the formation of cell pellets and subsequently incubated at 37 °C in a humidified atmosphere with low oxygen tension (5% O2) to retain their differentiated chondrocytic phenotype and to maximize the production of extracellular matrix (25). For inhibitor studies, chondrocyte pellets were weaned from the serum gradually by replacing the media every 3–4 days with decreasing concentrations of FBS (5, 2.5, and 0%). Chondrocytes were then cultured in pellet form for 3 weeks in the absence of serum to allow the accumulation of the proteoglycan- and collagen-containing extracellular matrix. Chondrocyte cell pellets were pre-incubated for 2 h with inhibitors prior to cytokine stimulation for 18 h. The level of sulfated glycosaminoglycan in the culture media and cartilage/pellet extracts was determined by the dimethylmethylene blue (DMMB) assay (26). Shark and whale chondroitin sulfate (Fluka Biochemika, Switzerland) was used as a standard. Cytotoxicity testing was performed for each of the inhibitors at the concentrations used in the pellet culture assays by measuring lactate levels in culture media as an indicator of cellular metabolism and viability using a kit from Sigma.

Isolation of RNA from Primary Cartilage Tissue and from Chondrocytes in Culture—RNA was isolated from human osteoarthritic articular cartilage samples obtained from patients (n = 18, mean age = 66.2 years, range 49–84 years) undergoing total knee replacement surgery (New England Bap-
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tist Hospital) or from nonosteoarthritic cartilage obtained from above-knee amputations (n = 10, mean age = 71.6 years, range 43–100) (Clinomics). The OA cartilage samples were obtained as whole joints within 2 h of surgery, and the articular cartilage was shaved from the joint surfaces taking great care to avoid any pannus, fibrotic tissues, subchondral bone, and other noncartilaginous regions of the joint (27). Nonosteoarthritic cartilage samples were obtained from individuals without a clinical diagnosis nor symptoms of OA, and the specimens were evaluated histologically to confirm the classification prior to inclusion in this study. Cartilage pieces were flash-frozen in liquid nitrogen and stored at −80 °C until processed for RNA isolation. The frozen cartilage was pulverized using a Spex Certiprep freezer mill (model 6750) at 15 Hz two times for 1 min each under liquid nitrogen. The frozen powdered cartilage was resuspended in 4 M guanidinium isothiocyanate (Invitrogen) containing 8.9 mM 2-mercaptoethanol and homogenized on ice with a Polytron homogenizer at maximum speed setting twice for 1 min each time, with a 1 min “rest” between homogenizations. The homogenate was centrifuged at 1500 × g for 10 min, and the supernatant was saved. The gelatinous pellet was resuspended in guanidinium isothiocyanate/2-mercaptoethanol and homogenized a second time as described above. The pellet was then discarded, and the two resulting supernatant fractions were combined and incubated with Triton X-100 (2% final concentration) and sodium acetate (pH 5.5, 1.5 M final concentration) sequentially for 15 min each. The samples were extracted once with an equal volume of acid phenol chloroform, pH 4.5, and twice with acid phenol, pH 4.5, phenol, pH 7.5, chloroform mix (1:1:1). RNA was subsequently precipitated by the addition of isopropl alcohol, and further purified using an RNeasy mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. RNA quantity and purity were measured by ultraviolet absorbance at A260/A280, and RNA quality was assessed by the RNA6000 assay using the Agilent BioAnalyzer 2100 (Palo Alto, CA). RNA yields averaged between 5 and 10 µg of total RNA per g of cartilage tissue.

For isolation of RNA from chondrocyte pellet cultures and from chondrocytes in monolayer culture, no pulverization was required. Pellets were digested with collagenase (2.5 mg/ml, Sigma), and RNA was subsequently prepared using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Primary chondrocytes and chondrocytic cell lines in monolayer culture were lysed by direct addition of TRIzol reagent followed by standard TRIzol RNA purification methodologies.

Microarray Analysis of Osteoarthritic and Normal Cartilage—Gene expression changes in RNA from lesional (n = 14) and adjacent nonlesional (n = 13) osteoarthritic cartilage compared with nonosteoarthritic cartilage (n = 10) were analyzed using the Human Genome U95Av2 (HG-U95Av2) GeneChip® Array (Affymetrix, Santa Clara, CA) for expression profiling. The HG-U95Av2 chip contains 25-mer oligonucleotide probes representing ~12,000 primarily full-length sequences (~16 probe pairs/sequence) derived from the human genome. For each probe that is designed to be perfectly complementary to a target sequence, a partner probe is generated that is identical except for a single base mismatch in its center. These probe pairs allow for signal quantitation and subtraction of nonspecific noise. RNA was extracted from individual articular cartilage tissue samples, converted to biotinylated cRNA, and fragmented according to the Affymetrix protocol. The fragmented cRNAs were diluted in 1 × MES buffer containing 100 µg/ml herring sperm DNA and 500 µg/ml acetylated bovine serum albumin and denatured for 5 min at 99 °C followed immediately by 5 min at 45 °C. Insoluble material was removed from the hybridization mixtures by a brief centrifugation, and the hybridization mixture was added to each array and incubated at 45 °C for 16 h with continuous rotation at 60 rpm. After incubation, the hybridization mixture was removed, and the chips were extensively washed and stained with streptavidin (R)-phycocerythrin (Molecular Probes, Eugene, OR) using the GeneChip® Fluidics Station 400 following the manufacturer’s specifications. The raw fluorescent intensity value of each transcript was measured at a resolution of 6 µm with a Hewlett-Packard Gene Array Scanner. GeneChip® software 3.2 (Affymetrix), which uses an algorithm to determine whether a gene is “present” or “absent,” as well as the specific hybridization intensity values or “average differences” of each gene on the array, was used to evaluate the fluorescent data. The average difference for each gene was normalized to frequency values by referral to the average differences of 11 control transcripts of known abundance that were spiked into each hybridization mixture according to the procedure of Hill et al. (28). First, the frequency of each gene was calculated and represents a value equal to the total number of individual gene transcripts per 10⁸ total transcripts. Transcripts which were called present by the GeneChip® software in at least one of the arrays for both arthritis and normal cartilage, were included in the analysis. Second, for comparison between arthritis and normal cartilage, a t test was applied to identify the subset of transcripts that had a significant (p < 0.05) increase or decrease in frequency values. Third, average fold changes in frequency values across the statistically significant subset of transcripts were required to be 2.0-fold or greater. These criteria were established based upon replicate experiments that estimated the intra-array reproducibility.

Quantitative RT-PCR (TaqMan®) —RNA for TaqMan® analysis (ABI PRISM 7700 sequence detection; PerkinElmer Life Sciences) was isolated from primary cartilage tissue as described above and then further purified with two more rounds of phenol/chloroform extraction followed by RNeasy (Qiagen) column binding and elution. To ensure the elimination of genomic DNA, RNA was treated with DNase (Qiagen) during RNeasy column purification (as recommended by the supplier), and following the RNA purification any residual genomic DNA was removed using DNA-free (Ambion, Austin, TX), following that manufacturer’s instructions.

For comparison of PKCζ and PKCζ mRNA levels in human OA chondrocytes, human primary chondrocytes were isolated as described above for bovine primary chondrocytes. The primary chondrocytes were plated in a 24-well format (2 × 10⁶ well) in DMEM/F-12 + 10% FBS + 100 units/ml penicillin, 100 µg/ml streptomycin for 2 days. The cells were lysed, and RNA was isolated using the RNeasy mini kit (Qiagen). 200 ng of RNA was used for each TaqMan assay in the TaqMan one-step RT-PCR method (Applied Biosystems). The human PKCζ and PKCζ probe/primer sets were “Assays on Demand” from
Oligonucleotide primers and fluorescent-labeled TaqMan probes for bovine ADAMTS-4, ADAMTS-5, and GAPDH cDNA sequences were designed using Primer Express 1.0 software (Applied Biosystems, Warrington, UK). Sequences for primers and probes were as follows: bADAMTS-4 forward, 5'-TGTGTTGCTGGGATGTT-3', reverse, 5'-GCACCAGGATGTTGGTGTG-3', and probe, 5'-FAM-CTGGCTCCCTTCAAAATATCGTACGGGA-TAMRA-3'; bADAMTS-5 forward, 5'-ATTTCCGGCTCCACGGAAGAT-3', reverse, 5'-TTCTGTGATTGGTTGCTAGG-3', and probe, 5'-FAM-ATTTACGCACCCCCACACTGTTCCA-TAMRA-3'; bGAPDH forward, 5'-AAAGTGGACATCGTCGCCAT-3', reverse, 5'-GACTGTGCGTGTGAACCTGC-3', and probe, 5'-FAM-TTCTACCATCATGGTCTACATGTTCCAGTATGATTCCA-TAMRA-3'. TaqMan PCR analysis was performed using the Applied Biosystems ABI Prism 7700 sequence detection system (TaqMan). PCRs for all samples were performed in duplicate (20 ng of DNase-treated purified total RNA) using TaqMan one-step PCR master mix reagent kit (Applied Biosystems) following the manufacturer's protocol. Thermocycler conditions comprised an initial reverse transcription step with incubation at 48 °C for 30 min, followed by AmpliTaq Gold enzyme activation at 95 °C for 10 min, and finally PCR amplification performed at 95 °C for 15 s and 60 °C for 1 min for 40 cycles. Threshold cycle (CT) values were obtained for ADAMTS-4 and ADAMTS-5, and the values were divided by CT values for GAPDH to obtain the relative expression level of aggrecanases normalized to GAPDH expression.

Phosphoprotein Analysis of PKCζ—For the chondrocyte cell line, T/C-28a2 cells (23) (kindly provided by Mary Goldring) were plated in a 24-well format in DMEM/F-12 + 10% FBS + 1% antimycotic antibiotic solution and grown to confluence. After the cells became confluent, they were changed to serum-free medium, allowed to adapt to serum-free conditions overnight, and were then stimulated with 20 ng/ml recombinant free medium, allowed to adapt to serum-free conditions overnight in 1% antimycotic antibiotic solution and grown to confluence.

Immunodetection of Aggrecan Cleavage Product—Detection of aggrecan cleavage sites in pellet culture conditioned media was performed using neoeptope monoclonal antibody Agg-C1 (anti-NITGE#, detects aggrecanase cleavage at aggrecan interglobular domain site (24)). Equal volumes of conditioned media from pellet cultures following incubation with or without cytokines and/or inhibitors were deglycosylated with chondroitinase ABC and keratanase (Calbiochem) and separated by 4–12% NOVEX Tris-glycine gels (Invitrogen). Subsequently, the samples were electrophoretically transferred to Hybond membrane (Amersham Biosciences) and incubated with Agg-C1 antibody overnight at 4 °C in TSA (50 mM Tris-Cl, pH 7.4; 0.2 M NaCl; 0.02% sodium azide). Unbound antibody was removed by washing the membrane in 1 × TSA buffer three times for 5 min, followed by incubation with goat anti-mouse IgG-alkaline phosphate conjugate secondary antibody (1:5000, Vector Laboratories) followed by detection using the ECL Western blotting detection kit (Amersham Biosciences). Western blots were visualized using a goat anti-mouse horseradish peroxidase conjugate followed by detection using the ECL Western blotting detection kit (Amersham Biosciences) and separated by 4–12% NOVEX Tris-glycine gels (Invitrogen). Subsequently, the samples were electrophoretically transferred to Hybond membrane (Amersham Biosciences) and incubated with Agg-C1 antibody overnight at 4 °C in TSA (50 mM Tris-Cl, pH 7.4; 0.2 M NaCl; 0.02% sodium azide). Unbound antibody was removed by washing the membrane in 1 × TSA buffer three times for 5 min, followed by incubation with goat anti-mouse IgG-alkaline phosphate conjugate secondary antibody (1:5000, Novagen) for 1 h at room temperature in 1 × TSA buffer. Following a second set of three 5-min washes in 1 × TSA, immunoreactive products were detected as described previously (28) by developing the Western blot with bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Promega), and the image was digitized using a Hewlett-Packard flatbed scanner.

Quantitation of Atypical PKC Protein Levels in Human Chondrocytes—Specific rabbit polyclonal antibodies against PKC ζ/λ and PKCζ were generated by immunizing rabbits with peptides spanning either residues 184–234 (PKC ζ/λ; GenBank™ accession number NM_008857.2) or 185–244 (PKCζ GenBank™ accession number NM_008860.2). Primary human chondrocytes were isolated from live cartilage using Pronase and collagenase digestion as described above for bovine cartilage. Whole cell lysates from these primary human OA chondrocytes (~150 µg per sample) were size-fractionated by 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane (Hybond ECL; Amersham Biosciences). The primary antibody reaction was performed overnight at 4 °C. Unbound antibodies were removed by washing the nitrocellulose membrane three times for 15 min in washing buffer (PBS, pH 7.4, 0.1% Tween 20) at room temperature. Subsequently, the membrane was incubated with secondary antibody (goat anti-rabbit horseradish peroxidase, 1:5,000; Dianova) for 2 h at room temperature followed by washing as described above. Antibodies were detected by chemiluminescence using ECL Western blotting detection reagents (Amersham Biosciences).
selected for resistance to puromycin. Cells that survived the selection were screened by a luciferase reporter assay (Promega) after IL-1β (10 ng/ml) induction. Puromycin-resistant T/C-28a2/NF-κB-luciferase cell lines were first selected for response to IL-1β induction with a minimum signal/background ratio of 5 in the luciferase reporter assay (Promega). The clones that passed this primary screen were further tested using TNF-α (5 and 20 ng/ml)/IL-1β (5 and 20 ng/ml). In this secondary screening, a T/C-28a2/NF-κB-luciferase clone was selected that showed the highest signal/background ratio and dose-dependent response to both TNF and IL-1 compared with other clones at the same cell density. Optimal conditions for using this cell line to test NF-κB response to cytokines ± inhibitors were determined empirically. The conditions found to be optimal were plating density of 30,000 cells/well in a 96-well format, 10 ng/ml IL-1β concentration, 25 ng/ml TNF-α concentration, 1 h of preincubation with inhibitors prior to cytokine induction, and a 3-h incubation with cytokines before luciferase assay. Cytotoxicity testing was performed for each of the inhibitors at the concentrations used in the NF-κB-luciferase assays either by measuring lactate levels in culture media as a measure of cellular metabolism and viability using a kit from Sigma, or by directly measuring cellular proliferation using the WST-1 assay (Roche Applied Science).

**Adenovirus Constructs and Infection Conditions**—The vectors used to produce adenovirus were replication-defective human adenovirus type 5 with complete deletion of the E1a and E1b regions and partial deletion of the E3 regions of the viral genome. Separate adenoviral expression constructs were created that contained cDNAs encoding full-length active human PKCζ (GenBank™ accession number Q05513); a mutant human PKCζ (DN−PKCζ) in which the alteration of a key residue in the ATP-binding site (K281W) results in a dominant negative kinase (29, 30); an NF-κB-luciferase reporter gene construct; and a green fluorescent protein virus as a transduction normalization standard (Ad-GFP). In experiments utilizing infection of primary bovine chondrocytes with the NF-κB-luciferase reporter virus, cells were infected 4 days prior to the experiment with equal m.o.i. (typically 100 m.o.i.) that resulted in 100% infectivity as determined by titrated Ad-GFP infections. These m.o.i. levels were tested and confirmed to be noncytotoxic using the lactate assay. All constructs (except the NFκB-luciferase reporter gene virus) expressed cDNAs under the control of the cytomegalovirus (CMV) promoter. These vectors were used to propagate recombinant viruses in human embryonic kidney cells (HEK293) (ATCC, Manassas, VA), which were then purified by two rounds of cesium chloride centrifugation (31). The purified virus was dialyzed in phosphate-buffered saline (PBS) and stored at −80 °C in 10% glycerol in PBS at a concentration of 10⁶ plaque-forming units/μl. Adenovirus were generated, purified, and titered by ViraQuest Inc. (North Liberty, IA).

**RESULTS**

**Protein Kinase Cζ Is Transcriptionally Up-regulated in Human Osteoarthritic Cartilage**—Gene expression changes in articular chondrocytes from lesional (n = 14) and adjacent nonlesional (n = 13) osteoarthritic cartilages compared with nonosteoarthritic cartilages (n = 10) were analyzed using Affymetrix GeneChip® U95Av.2 arrays. Analysis of these data to identify the global transcriptional changes in articular chondrocytes that were associated with osteoarthritis is beyond the scope of this paper, and will be published separately.3 However, a focused analysis of the data was performed to specifically identify OA-associated expression changes in protein kinase C family members. Probe sets for nine PKC isoforms were represented on the U95Av.2 array as follows: α, β, γ, δ, ε, θ, η, ι, and ζ. Only four of these PKC isoforms, δ, ι, θ, and ζ, were judged to be present by virtue of their hybridization intensity levels and the consistent hybridization performance of their probes on the arrays. Of these four PKC isoforms, only PKCζ and PKCζ exceeded an empirically determined signal intensity threshold of 50 signal units allowing reliable quantitation of their transcript levels on the gene chips, and only PKCζ appeared to be transcriptionally altered in OA articular cartilage compared with normal articular cartilage (Fig. 1A and data not shown). Transcript levels for PKCζ, the only other human atypical PKC isoform, were weakly detected on the gene chips and did not appear to show disease-related expression changes, but it was expressed at levels too low for accurate quantitation by this methodology. When the raw gene chip signal intensity for PKCζ hybridization was converted to normalized mRNA quantities (in parts per million) using an intrinsic standard curve on the chips (28), the PKCζ transcript levels were found to be ~2.5–3.5-fold higher in the nonlesional and lesional OA cartilage samples compared with normal cartilage (Fig. 1B). The up-regulation of PKCζ mRNA in the lesional OA samples reached statistical significance (p < 0.004), but variability in the PKCζ values in the nonlesional samples limited the significance of the up-regulation in those samples on the gene chips. To confirm the gene chip results and to increase the sensitivity of the transcriptional analysis, PKCζ transcript levels were measured in RNA from these same human samples by TaqMan quantitative RT-PCR (Fig. 1C). These TaqMan data supported the gene chip results, confirming that PKCζ mRNA was up-regulated 3.5-fold in nonlesional OA articular cartilage (p < 0.002 versus normal cartilage) and 2.0-fold in lesional OA articular cartilage (p < 0.02 versus normal cartilage).

**PKCζ Is the Predominant Atypical PKC Isoform in Articular Cartilage**—Because PKCζ shares a high degree of sequence similarity with PKCζ (72% amino acid identity overall and even greater within the catalytic domain (32, 33)), differentiating between PKCζ and PKCζ using most available biochemical reagents is difficult or impossible. Therefore, we were interested in determining how PKCζ transcript levels compared with PKCζ in an effort to judge the potential extent or proportion of the functional contribution of PKCζ in subsequent experiments. Because PKCζ mRNA levels were too low for accurate quantitation in the gene chip transcriptional profiling, more sensitive TaqMan assays were performed with probe/primer sets designed to distinguish PKCζ from PKCζ. These assays were first calibrated with known input amounts of PKCζ and PKCζ cDNA to allow direct comparison of transcript abun-

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3 A. J. Dorner, E. A. Morris, and E. R. LaVallie, manuscript in preparation.
dence between genes (data not shown). The correction factor based upon assay efficiencies in these calibration assays was 0.988 (PKCζ = PKCζ/0.988). RNA was isolated from articular chondrocytes from three separate donors with end stage OA, and PKCζ and PKCδ transcript levels were measured in each donor sample by TaqMan Q-PCR. The threshold cycle (Ct) value for PKCζ for PKCζ abundance 9 cycles lower than for PKCδ (Fig. 2A). In Fig. 2B, transcript abundance for each gene was converted to TaqMan units (raw Ct value normalized by comparison to GAPDH in the same samples), and the normalized value was corrected for assay efficiency by calculation of absolute transcript levels using the cDNA calibration curves. The result showed that the abundance of PKCζ mRNA in human OA articular cartilage was more than 800 times greater than PKCδ, which was detectable but present at consistently low levels in all three of the OA articular cartilage samples (Fig. 2B).

To determine whether the protein level for PKCζ in chondrocytes was as predominant compared with PKCδ as the mRNA levels would indicate, antibodies capable of distinguishing between PKCζ and PKCδ (“Experimental Procedures”) were utilized. Cell lysates from human primary OA chondrocytes were run on separate 10% SDS-polyacrylamide gels and immunoblotted with either anti-PKCζ antibody, anti-PKCδ antibody, or the C-20 polyclonal antibody that recognizes both isoforms. These Western blots, shown in Fig. 2C, clearly show that PKCζ protein accounts for virtually all of the detectable aPKC protein in the human chondrocyte cell lysates, supporting the mRNA abundance data and providing additional evidence that PKCζ is not present to any significant extent in articular chondrocytes, whereas PKCδ is relatively abundant.

PKCζ Is Activated by IL-1 Signaling in Chondrocytes—The role of IL-1 in the initiation of extracellular matrix destruction in chondrocytes has been well established (24, 34–37). IL-1 has been shown to trigger the phosphorylation of PKCζ in some cell types (38, 39). To investigate whether PKCζ may be downstream of IL-1 signaling in chondrocytes, a human immortalized chondrocyte cell line T/C-28a2 (23) and primary bovine articular chondrocytes were treated separately in culture with 20 ng/ml IL-1β or IL-1α, respectively. We have shown previously that at equivalent doses the recombinant human IL-1α is more potent than recombinant human IL-1β on bovine chondrocytes in terms of activation of NF-κB and degradation of matrix proteoglycan (24), whereas human- and porcine-derived chondrocytes show greater response to IL-1β.4 Because IL-1α and IL-1β signal through the same cell-surface receptor and elicit the same downstream responses (40), we assumed that the differences in potency reflected differences in cross-species binding affinities for the two isoforms of IL-1 to the cognate receptors and therefore chose the most active cytokine for each system. At various time points up to 10 min after addition of IL-1, cultures were lysed and the cell lysates were immunoprecipitated using an antibody to p62 (41). p62 is a scaffolding protein known to associate with PKCζ when it is activated by upstream protein kinases (42, 43). Thus, immunoprecipitation of p62 provides enrichment for PKCζ that has been phosphorylated by upstream kinases. p62 protein, along with proteins from the cell lysates that were bound to it, was recovered and run on an SDS-polyacrylamide gel, and a Western blot was performed either with an antibody that specifically binds PKCζ when it is phosphorylated at threonine 410 (Fig. 3, top panels of A and C) or with an antibody raised to a peptide representing the carboxyl-terminal 20 amino acids of PKCζ that binds to PKCζ regardless of its phosphorylation state (Fig. 3, bottom panels of A and C). Phosphorylation of PKCζ at Thr410 is known to be due to the activity of PDK-1, and this phosphorylation event initiates the activation of PKCζ enzymatic activity (44). In the T/C-28a2 cell line, basal levels of phospho-Thr410 PKCζ were low (Fig. 3, A and B). Upon addition of IL-1β, phosphorylation of PKCζ at the Thr410 position was significantly increased after

4 E. R. LaVallie and M. Arai, unpublished results.
FIGURE 2. **Comparison of PKCζ and PKCε mRNA and protein levels in human articular chondrocytes.** Quantitative RT-PCR assays (TaqMan®) were used to evaluate the relative abundance of mRNA for PKCζ and PKCε in human osteoarthritic cartilage samples. A, threshold cycle values (mean ± S.D.) for PKCζ and PKCε in articular cartilage RNA from three different human donors with end stage OA. B, “TaqMan units” were calculated for each transcript by comparing Ct values for each sample (assayed in triplicate) to a standard curve consisting of known quantities of cDNA for each gene. The extrapolated transcript abundance from the standard curve was then normalized to an internal standard (GAPDH) for each assay. The calculated values are shown within the bar graph because the PKCζ levels were too low for graphing on this scale. C, immunoblot analysis of primary chondrocyte cell lysates from two different (“H3” and “H4”) human donors with end stage OA. Identical blots were prepared from 10% SDS-polyacrylamide gels loaded with 150 μg of cell lysate protein per lane, and the immobilized proteins were individually subjected to Western blotting with either a rabbit polyclonal antibody that recognizes both PKCζ and PKCε (“aPKC C-20”, left panel), or with rabbit polyclonal antibodies that specifically recognize PKCζ (middle panel) or PKCε (right panel). Data shown for these two donors typify the results seen for additional samples that were tested.

Increased cleavage of aggrecan (the most abundant cartilage extracellular matrix proteoglycan) at specific sites that are characteristic of OA (45–47). Both ADAMTS-4 and ADAMTS-5 are expressed by articular chondrocytes (48), and although there has been some discrepancy in the literature, there is cumulative evidence that both are transcriptionally up-regulated when chondrocytes are treated with either IL-1 or TNF-α (24, 48, 49). Using a primary bovine articular chondrocyte pellet culture assay system that we devised (24), the effect of PKC inhibitors and an NF-κB inhibitor on TNF-α- and IL-1α-mediated induction of ADAMTS-4 and ADAMTS-5 was tested. Bovine chondrocyte three-dimensional pellet cultures surrounded by self-synthesized extracellular matrix were pretreated with the following inhibitors: 10 μM RO 31-8220 (3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide; bisindolylmaleimide IX); 40 μM myristoylated PKCζ pseudosubstrate (PS) peptide (50, 51); 100 μM SN50, a cell-permeable peptide inhibitor of NF-κB nuclear translocation; and an equivalent concentration of the control peptide SN50M, a mutated, inactive derivative of SN50 (19). RO 31-8220 reportedly inhibits all PKC isoforms with varying potency and also inhibits mitogen-activated protein kinase phosphatase-1 (MKP-1) expression, induces c-Jun expression, and activates Jun amino-terminal kinase (15). Following pretreatment with inhibitors (control cultures received no inhibitor pretreatment), TNF-α or IL-1α (or no cytokine) was added to the cultures. After 18 h, cytokine-mediated matrix destruction was assessed by measuring the amount of total proteoglycan released from the pellet cultures to the conditioned media by DMBM assay (24). Fig. 4A shows that both TNF-α and IL-1α significantly increased proteoglycan degradation in the pellet cultures in the absence of inhibitors, resulting in ~4-fold more proteoglycan released to the media when compared with the “no cytokine” control. RO 31-8220 treatment without addition of cytokines had no effect on proteoglycan release; however, addition of RO 31-8220 prior to TNF-α or IL-1α effectively blocked the cytokine-mediated proteoglycan degradation seen in the control cultures (p < 0.01). Addition of the PKCζ PS peptide also resulted in significant reduction of TNF-α and IL-1α-mediated proteoglycan release (p < 0.01), as did the NF-κB blocker SN50 but not the SN50M negative control peptide (Fig. 4A). Evaluation of proteoglycan levels remaining in the pellet showed that the proteoglycan...
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released to the conditioned media upon cytokine treatment resulted in a concomitant decrease in pellet proteoglycan content, and inhibitor pretreatment preserved the proteoglycan content of the pellets (data not shown). Therefore, the observed decrease in cytokine-mediated proteoglycan release to the conditioned media resulting from inhibitor pretreatment of pellet cultures appeared to be attributable to decreased proteoglycan degradation and not to decreased proteoglycan synthesis.

Aggrecanase neoepitope Western analysis of conditioned media from the different pellet cultures in the experiment shown in Fig. 4A revealed that the proteoglycan fragments released from IL-1- or TNF-treated chondrocyte pellet cultures in the absence of inhibitors contained a substantial amount of aggrecanase cleavage products that were readily detectable on Agg-C1 neoepitope Western blots (Fig. 4B, lanes 1 and 2). These IL-1- and TNF-induced aggrecanase cleavage products were markedly decreased in abundance by pretreatment with RO 31-8220, PKCζ pseudosubstrate peptide, or the SN50 NF-κB blocking peptide in a manner that closely mirrored the DMMB data in Fig. 4A, suggesting that blocking PKCζ or NF-κB activity prior to cytokine treatment resulted in inhibition of aggrecanase expression and activity.

To test this assumption, total RNA was extracted from the pellet cultures at the end of the culture period, and quantitative RT-PCR was performed using probe/primer sets designed to bovine ADAMTS-4 (Agg-1) and bovine ADAMTS-5 (Agg-2) mRNA sequences (24). Bovine GAPDH was used as a normalization control. Both TNF-α and IL-1α treatment induced Agg-1 and Agg-2 mRNA levels in these chondrocyte cultures (Fig. 4C and D). Agg-1 mRNA induction by TNF-α and IL-1α was significantly suppressed by RO 31-8220 and SN50 ($p < 0.01$), and the PKCζ PS peptide also appeared to reduce Agg-1 mRNA levels, but the effect did not reach statistical significance (Fig. 4C). However, up-regulation of Agg-2 mRNA by TNF and IL-1 in the primary chondrocyte cultures was significantly suppressed by all three inhibitors (Fig. 4D), demonstrating that PKCζ inhibition can effectively block induction of aggrecanase expression in chondrocytes by these inflammatory cytokines. Thus, the proteoglycan degradation caused by exposure of primary chondrocytes to IL-1 or TNF involves PKCζ-dependent aggrecanase up-regulation that results in accumulation of aggrecan fragments in the conditioned media that are cleaved at the Glu373–Ala374 site in the interglobular domain of aggrecan. Because aggrecan is the predominant proteoglycan in cartilage matrix (52), these data support a model in which suppression of cytokine-mediated aggrecanase induction by inhibition of PKCζ activity is the mechanism by which proteoglycan is preserved in this system.

To further explore the effects of specific inhibition of PKCζ, primary bovine chondrocyte pellet cultures were pretreated with increasing concentrations of the myristoylated (cell-permeable) PKCζ PS peptide prior to addition of 10 ng/ml IL-1α and overnight (18 h) incubation. Doses of peptide as low as 10 μM significantly ($p < 0.05$) reduced induction of proteoglycan degradation by IL-1α, and doses of 20 μM or more of the PKCζ PS peptide led to even more significant reduction ($p < 0.01$) of the IL-1α effect on proteoglycan release from the pellet cultures (Fig. 5A). The inhibition of cytokine-induced proteoglycan release by the PKCζ PS peptide was not attributable to decreased synthesis of proteoglycan due to cytotoxicity, because lactate assays on conditioned media from these same pellet cultures showed no decrease in cellular metabolism (Fig. 5B). In addition, evaluation of the proteoglycan content of the cell pellets following papain digestion at the end of the experiment showed that total proteoglycan synthesis was not impaired (data not shown). In this same cytokine-induced chondrocyte proteoglycan release assay using TNF-α, comparison of the myristoylated PKCζ PS peptide to a nonmyristoylated PKCζ PS peptide with the identical sequence, and to a myristoylated peptide containing the pseudosubstrate sequence of PKCζ, revealed that the inhibitory effect was specific to PKCζ and required cell permeability (Fig. 5C). An additional myristoylated control peptide containing the same amino acid content as the PKCζ PS peptide but with the order of the amino acids scrambled has also been repeatedly tested in this assay and showed no inhibitory effect on cytokine-induced matrix degradation (data not shown).
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An NF-κB-luciferase reporter gene construct was stably integrated into the immortalized human costal chondrocyte cell line T/C-28a2 (23), and a reporter cell line was developed to evaluate whether PKCζ was responsible (directly or indirectly) for the activation of NF-κB in chondrocytes by IL-1α and TNF-α, as it is in some other cell types (43, 53–56). The resulting cell line was selected to be highly responsive to IL-1α and TNF-α treatment as judged by its luciferase expression via NF-κB-mediated transcription (see “Experimental Procedures”). As shown in Fig. 5D, the induction of the NF-κB-luciferase reporter gene by either IL-1α or TNF-α in this human chondrocytic cell line was blocked by both triptolide (an NF-κB inhibitor (20)) and by the myristoylated PKCζ PS peptide inhibitor (p < 0.01), but not by the nonmyristoylated PKCζ PS peptide nor by the myristoylated PKCα/β PS peptide. These experiments provide evidence that TNF-α and IL-1α elicit comparable effects in bovine primary chondrocytes with regard to PKCζ-dependent activation of NF-κB-mediated transcription and proteoglycan degradation, and that the T/C-28a2 human chondrocyte cell line responds in a very similar fashion to primary bovine chondrocytes.

TGF-β and IL-1 Induction of NF-κB and Resulting Proteoglycan Degradation in Chondrocytes Is Blocked by Pan-PKC Inhibitors but Not by an Atypical-Sparing PKC Inhibitor—

Additional PKC inhibitors with different specificities were used with the T/C-28a2 human chondrocyte cell line in an effort to further implicate the ζ isoform as the responsible protein kinase C family member in the pathways by which TNF and IL-1 signal through NF-κB. Bisindolylmaleimide I (BIS) inhibits all PKC isoforms with a potency rank order of cPKC > nPKC > aPKC (16, 57). IL-1β induction of the NF-κB-luciferase reporter gene in the T/C-28a2 cell line was inhibited by almost 80% by 12.5 μM BIS, with less inhibition noted with decreasing concentrations of BIS (Fig. 6A). This level of inhibition is consistent with the reported IC₅₀ of 5.8 μM for BIS on PKCζ, which is almost 300-fold higher than the IC₅₀ for BIS on cPKCs (0.02 μM) and more than 30-fold higher than the IC₅₀ for BIS on nPKCs (58). Gö 6976 report-edly is also most potent against the Ca²⁺-requiring (convensional) PKC isoforms and is less potent against the nPKCs and aPKCs, with an IC₅₀ for PKCζ of >10 μM (21, 59). Gö 6976 showed some inhibition of both IL-1 and TNF induc-
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Sphingomyelinase-induced Transcription via NF-\(\kappa\)B in Chondrocytes Is Dependent on PKC\(\xi\)—Ceramide is a second messenger that is liberated by hydrolysis of sphingomyelin (a cell membrane-derived sphingolipid) by sphingomyelinases (61). Sphingomyelinase (SMase) activity has been shown to be up-regulated in some cell types by treatment with TNF or IL-1 (62–65). Furthermore, ceramide is capable of directly activating PKC\(\xi\) without stimulation of upstream signal transduction components (66–68). Direct treatment of cells with sphingomyelinase has been shown to increase intracellular ceramide levels resulting in the activation of PKC\(\xi\) (66, 69). Therefore, if PKC\(\xi\) is an important signaling component of the NF-\(\kappa\)B pathway in chondrocytes, it would be expected that increasing ceramide levels within these cells would result in NF-\(\kappa\)B activation, and this activation should be blocked by addition of PKC inhibitors. Fig. 7 shows the results of such an experiment, in which primary bovine chondrocytes expressing an NF-\(\kappa\)B-luciferase reporter gene were induced with either 10 or 40 milliunits of SMase or TNF, cells received either no pretreatment or a 1-h pretreatment with the pan-PKC inhibitor BIS (20 \(\mu\)M), the atypical PKC-sparing calphostin C (CalC, 100 nM), or to a myristoylated peptide representing the pseudosubstrate sequence for PKC\(\alpha/\beta\) (Myr PKC\(\alpha/\beta\) PS, 40 \(\mu\)M) and to a myristoylated peptide representing the pseudosubstrate sequence for PKC\(\zeta\) (Non-Myr PKC\(\zeta\) PS, 40 \(\mu\)M) in the primary bovine chondrocyte pellet culture proteoglycan release assay. D, the T/C-26a2 cell line with NF-\(\kappa\)B-luciferase reporter stably integrated was pretreated with the same pseudosubstrate peptides as in C (50 \(\mu\)M each), compared with the NF-\(\kappa\)B inhibitor triptolide (50 \(\mu\)M). Luciferase levels were measured after 3 h of incubation following cytokine addition. Control cultures without inhibitor added were used to define 100% NF-\(\kappa\)B-luciferase activity. Bars represent the mean \(\pm\) S.D. of replicate experiments and significant differences were determined by comparisons using the Student's t test.

FIGURE 5. Proteoglycan degradation and NF-\(\kappa\)B activation mediated by either IL-1 or TNF is blocked by a PKC\(\xi\) pseudosubstrate peptide inhibitor in a dose-dependent manner. A and B, primary bovine chondrocyte pellet cultures were pretreated with increasing concentrations of myristoylated PKC\(\xi\) pseudosubstrate peptide inhibitor for 2 h prior to the addition of IL-1 or TNF to the culture. After 48 h, pellet culture conditioned media were harvested and assayed for proteoglycan release by DMMB assay (A) or for lactate levels (B). C, the myristoylated PKC\(\xi\) pseudosubstrate peptide (Myr PKC\(\xi\) PS, 40 \(\mu\)M) was compared with a nonmyristoylated version of the same peptide (Non-Myr PKC\(\xi\) PS, 40 \(\mu\)M) and to a myristoylated peptide representing the pseudosubstrate sequence for PKC\(\alpha/\beta\) (Myr PKC\(\alpha/\beta\) PS, 40 \(\mu\)M) in the primary bovine chondrocyte pellet culture proteoglycan release assay. D, the T/C-26a2 cell line with NF-\(\kappa\)B-luciferase reporter stably integrated was pretreated with the same pseudosubstrate peptides as in C (50 \(\mu\)M each), compared with the NF-\(\kappa\)B inhibitor triptolide (50 \(\mu\)M). Luciferase levels were measured after 3 h of incubation following cytokine addition. Control cultures without inhibitor added were used to define 100% NF-\(\kappa\)B-luciferase activity. Bars represent the mean \(\pm\) S.D. of replicate experiments and significant differences were determined by comparisons using the Student's t test.

Inhibition of NF-\(\kappa\)B-luciferase activity, but it was no more potent in this assay than BIS, despite the fact that Go6976 has an IC\(_{50}\) of 4-fold lower than BIS on cPKCs (21). Calphostin C is a compound that competes for the diacylglycerol/phorbol ester-binding site in the regulatory domain of the conventional and novel PKCs and competitively inhibits their activity (17). Because the atypical PKCs (\(\xi\) and \(\eta\)) lack this domain, their activity is unaffected by calphostin C. Calphostin C was totally ineffective at blocking IL-1 or TNF induction of NF-\(\kappa\)B (Fig. 6A), even at a concentration 100 times higher than its IC\(_{50}\) for cPKC and nPKC (17). Similar results were obtained in the bovine primary chondrocyte pellet culture assay (Fig. 6B). Increased degradation and release of extracellular matrix proteoglycan by addition of TNF-\(\alpha\) was totally blocked in this assay by 40 \(\mu\)M BIS, whereas calphostin C had no effect at concentrations up to 100 \(\mu\)M. Similar data were obtained in bovine and porcine chondrocytes treated with either IL-1 or TNF-\(\alpha\), even at calphostin C concentrations well above the IC\(_{50}\) for cPKC and nPKC (data not shown). Previous studies have shown that calphostin C is capable of inhibiting PKC\(\alpha\) activation in primary chondrocytes (18), and 100 \(\mu\)M calphostin C significantly inhibited proteoglycan synthesis induced by CCN2 in the chondrocytic cell line HCS-2/8 (60), proving that calphostin C is active on chondrocytes in culture. These data strongly implicate an atypical PKC as a necessary component of IL-1 and TNF signal transduction to NF-\(\kappa\)B in chondrocytes.
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A. 100

Inhibitor Concentrations

B.

Effect of Expression of a Dominant Negative (K281W) PKCζ Mutant on NF-κB Signaling in Chondrocytes—A kinase-defective mutant form of PKCζ was constructed in which a key residue in the ATP-binding region of the catalytic domain (lysine 281) was changed to a tryptophan. This mutation has been shown to create a dominant negative form of PKCζ (DN-PKCζ) that specifically and nonproductively competes for components in the signaling pathway, resulting in a specific competitive inhibitor for the kinase (29, 30, 43). The DN-PKCζ cDNA was placed under the control of a CMV promoter in an adenoviral vector, and adenovirus stocks were generated as described previously (24). Primary bovine chondrocytes were prepared and infected with an equal m.o.i. of viral stocks expressing either full-length active human PKCζ (FL-PKCζ), the DN-PKCζ, or an “empty” control virus without a cDNA inserted downstream of the CMV promoter. In some experiments, comparisons were made to a co-infected GFP-expressing adenovirus instead of the empty control virus, with comparable results (data not shown). These viruses were co-infected in combination with an NF-κB-luciferase reporter virus. After 4 days in culture to allow time for viral expression of the PKCζ constructs, cultures were treated with either IL-1α, with two different concentrations of TNF-α, or were not treated with any cytokine. As shown in Fig. 8, expression of FL-PKCζ resulted in

FIGURE 6. TNF and IL-1 induction of NF-κB and resulting proteoglycan degradation is blocked by pan-PKC inhibitors but not by an atypical sparing PKC inhibitor. A, the T/C-28a2 NF-κB-luciferase reporter cell line was pretreated for 1 h with increasing concentrations of either Go6976, calphostin C (0.6 μM-5 μM); BIS (1.6 μM-12.5 μM); triptolide (6.3 nM-50 nM) prior to addition of either IL-1β (10 ng/ml) or TNF-α (25 ng/ml). All compounds were shown to be noncytotoxic on these cells in the concentration ranges used in these assays by WST-1 proliferation assays. Luciferase expression was measured by luciferase assays on cell lysates 3 h after addition of cytokines. Control cultures without inhibitor added were used to define 100% NF-κB-luciferase activity. B, bovine pellet cultures received either no inhibitor (none) or were treated with BIS (40 μM) or with increasing concentrations of calphostin C (25-100 nM) prior to addition of 100 ng/ml TNF-α. Proteoglycan degradation was measured 18 h later using the DMMB assay on the culture conditioned media. Values represent the mean ± S.D. of three replicate experiments, and p values were derived from comparisons using the Student’s t test.
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A small but consistent increase in the basal levels of luciferase activity (in the absence of cytokine addition) when compared with the empty control virus. In some experiments this trend reached statistical significance, but in most experiments it did not (data not shown). No significant increase in cytokine-induced NF-κB-luciferase activity was observed when PKCζ was overexpressed, suggesting that normal intracellular levels of PKCζ protein are not limiting. However, expression of DN-PKCζ consistently resulted in significant attenuation of both IL-1α and TNF-mediated induction of NF-κB-luciferase expression when compared with the control virus (p < 0.002 for 0.1 ng/ml TNF-α versus control and p < 0.001 for 0.1 ng/ml IL-1α and 1 ng/ml TNF-α versus control). Comparable results were seen in similar experiments performed in primary human articular chondrocytes (data not shown). These data provide further evidence that PKCζ is a key mediator of both IL-1 and TNF signaling through NF-κB in chondrocytes.

DISCUSSION

The initial intent of these studies was to identify genes that display dysregulated expression in human articular chondrocytes from osteoarthritic cartilage when compared with non-osteoarthritic cartilage by transcriptional profiling on Affymetrix GeneChip microarrays. Cartilage is well suited for this type of transcriptional analysis, because the tissue contains only a single cell types (chondrocytes), and there are no blood vessels, nerves, or other cell types to contribute RNA that could confound the results. This is especially relevant because it is believed that the pathological changes in OA cartilage are manifested primarily by dysregulated gene expression in the chondrocytes (70).

Many genes were detected on the gene chips that displayed significantly altered transcript levels in OA cartilage compared with normal cartilage. The focus of this paper is one of those genes, the intracellular serine/threonine kinase PKCζ. A search for PKC family members in the human OA articular cartilage transcriptional profiling data revealed that PKCζ was the only PKC family member that showed altered expression in OA cartilage, and in fact only one other family member (PKCδ) was expressed at quantifiable levels on the gene chips. Many studies have demonstrated that PKCζ is involved in the pathway for activation of the NF-κB transcriptional factor (29, 30, 38, 41, 43, 53–55, 66, 67, 71, 72) as well as in the MAPK signaling cascade (71, 73). The NF-κB pathway was of particular interest to us because certain inflammatory cytokines thought to be intrinsically linked to OA pathophysiology, especially TNF and IL-1, are known to cause activation of NF-κB and result in increased expression of catabolic factors with destruction of articular cartilage that is characteristic of the OA disease process. In our studies, treatment of chondrocyte pellet cultures with RO 31-8220 (which has been shown to act as an activator of MAPK and c-Jun (15)) did not appear to cause an increase in proteoglycan degradation in the absence of TNF or IL-1. In addition, calphostin C has been shown to be capable of activating AP1 and inducing c-Jun transactivation (18), but treatment of chondrocyte pellet cultures with calphostin C in the absence of cytokines did not increase proteoglycan release. These results pointed us to the NF-κB pathway to evaluate a potential role of PKCζ in chondrocytes.

Recently, the characterization of a PKCζ knock-out (KO) mouse has been described (54). This KO mouse appeared to be grossly normal, but at the cellular and molecular level it displayed defects in NF-κB signaling of varying severity depending on the cell type. However, the impact of a lack of PKCζ on NF-κB signaling in chondrocytes from this KO mouse was not...
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reported. A possible explanation for the variability of the NF-κB phenotype in different tissues in the PKCζ KO mouse was that cell types that normally expressed higher levels of PKCζ might show a greater impact in NF-κB signaling (54, 55). Another possible explanation was that PKCζ might compensate for loss of PKCζ to varying degrees in different cell types. A third and very significant possibility arose after subsequent publications revealed that the gene disruption strategy that was used to generate the PKCζ KO allele still allowed the expression of a truncated, constitutively active form of PKCζ (called PKMζ) from an alternative, intronic promoter (74, 75). This alternative promoter was reported to be brain-specific; however, we have found PKMζ to be expressed in cartilage and in some other tissues by quantitative RT-PCR analysis. Therefore, the impact of PKCζ activity on NF-κB signaling may be even more important (and perhaps even essential) in more cell types than the initial analysis of this PKCζ KO mouse suggested.

The experiments described here utilized various PKC activators and inhibitors in chondrocyte cell-based assays to confirm the essential role of an atypical PKC in activation of NF-κB by TNF and IL-1 in this cell type. Importantly, these data showed that atypical PKC activity is not only essential for chondrocytic NF-κB activation, but also that inhibition of atypical PKC activity results in protection from the degenerative effects of TNF and IL-1 signaling on extracellular matrix in articular chondrocyte cultures. However, from our data it was difficult to conclusively pinpoint PKCζ as the responsible atypical isoform, and a role for PKCζ could not be definitively ruled out. The pseudosubstrate sequence of PKCζ is identical to the pseudosubstrate sequence of PKCε, so it would be expected that the myristoylated PKCζ pseudosubstrate peptide inhibitor used in this study would also inhibit PKCζ. Antibody reagents to phospho-PKCζ and total PKCζ also bind PKCζ, as does p62, so immunoprecipitation of p62 prior to phosphoprotein Western analysis does not help to distinguish between atypical isoforms (42). Also, it is possible that the dominant negative PKCζ construct used in these studies may interfere with activity as well as with activity. However, our findings that PKCζ is expressed at extremely low levels in chondrocytes and does not appear to be dysregulated in OA cartilage suggested to us that even if PKCζ was capable of functionally substituting for PKCζ, the large disparity in expression levels for the two isoforms in chondrocytes meant that PKCζ activity probably accounted for most if not all of the effect that we saw in our studies.

A recent publication by Soloff et al. (10) has provided important evidence to support this assumption. These investigators provided compelling evidence that the mouse ortholog of PKCζ (called PKCα) is not involved in the NF-κB signal transduction pathway. This group created a PKCα KO mouse that exhibited an embryonic lethal phenotype prior to day 9.5, which prevented the study of cells from the adult mouse. However, they were able to select for PKCA-deficient embryonic stem cells from PKCA+/− precursors and created immortalized PKCA-deficient mouse embryonic fibroblasts in which to study the effects of the loss of PKCα on signaling pathways. These PKCA-deficient fibroblasts showed no defect in NF-κB activation, as judged by the unimpaired degradation of IκBα and induction of a NF-κB-luciferase reporter construct in response to TNF treatment in these cells (10). It is important to note that Leitges et al. (54) had shown previously that TNF-treated mouse embryonic fibroblasts from the PKCζ KO mouse were one cell type that showed severe impairment of NF-κB signaling compared with PKCζ+/− mouse embryonic fibroblasts, providing a direct comparison of PKCζ versus PKCA contributions to NF-κB signaling in the same cell type.

Based upon these and our own observations, we conclude that PKCζ is the atypical PKC isoform that serves as a critical link to NF-κB activation by TNF and IL-1 in chondrocytes. In this regard, PKCζ represents a potentially important disease-associated gene responsible for OA cartilage destruction and an attractive therapeutic target. Orally active, small molecule inhibitors of PKCζ might potentially provide an attractive and powerful therapeutic option for the chronic treatment of OA, because such an approach may serve to block a common signaling constituent for multiple inflammatory cytokines as well as interrupt the synthesis of other mediators of both OA and rheumatoid arthritis that lie downstream of NF-κB (76).

Acknowledgments—We thank Debra Pittman and Helen Palmer for assistance in adenovirus preparation, Dr. Doug Harnish for the NF-κB-luciferase reporter gene adenovirus, and Drs. Shelley Russek, Susan Leeman, Chris Pierce, and Mary Goldring for guidance and helpful discussions.

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