Journal of Cellular Biochemistry 69:392–413 (1998)

Transcriptional Induction of Cyclooxygenase-2 Gene by Okadaic Acid Inhibition of Phosphatase Activity in Human Chondrocytes: Co-Stimulation of AP-1 and CRE Nuclear Binding Proteins

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Abstract The involvement of serine/threonine protein phosphatases in signaling pathways that control the expression of the cyclooxygenase-2 (COX-2) gene in human chondrocytes was examined. Okadaic acid (OKA), an inhibitor of protein phosphatases 1 (PP-1) and 2A (PP-2A), induced a delayed, time-dependent increase in the rate of COX-2 gene transcription (runoff assay) resulting in increased steady-state mRNA levels and enzyme synthesis. The latter response was dose dependent over a narrow range of 1–30 nmol/L with declining expression and synthesis of COX-2 at higher concentrations due to cell toxicity. The delayed increase in COX-2 mRNA expression was accompanied by the induction of the proto-oncogenes c-*jun, jun*B, *jun*D, and c-*fos* (but not *Fos*B or Fra-1). Increased phosphorylation of CREB-1/ATF-1 transcription factors was observed beginning at 4 h and reached a zenith at 8 h. Gel-shift analysis confirmed the up-regulation of AP-1 and CRE nuclear binding proteins, though there was little or no OKA-induced nuclear protein binding to SP-1, AP-2, NF-κB or NF-IL-6 regulatory elements. OKA-induced nuclear protein binding to ^{32}P-CRE oligonucleotides was abrogated by a pharmacological inhibitor of protein kinase A (PKA), KT-5720; the latter compound also inhibited OKA-induced COX-2 enzyme synthesis. Calphostin C (CalC), an inhibitor of PKC isoenzymes, had little effect in this regard. Inhibition of ^{32}P-CRE binding was also observed in the presence of an antibody to CREB-binding protein (265-kDa CBP), an integrator and coactivator of cAMP-responsive genes. The binding to ^{32}P-CRE was unaffected in the presence of excess radioinert AP-1 and COX-2 NF-IL-6 oligonucleotides, although a COX-2 CRE-oligo competed very efficiently. ^{32}P-AP-1 consensus sequence binding was unaffected by incubation of chondrocytes with KT-5720 or CalC, but was dramatically diminished by excess radioinert AP-1 and CRE-COX-2 oligos. Supershift analysis in the presence of antibodies to c-Jun, c-Fos, JunD, and JunB suggested that AP-1 complexes were composed of c-Fos, JunB, and possibly c-Jun. OKA has no effect on total cellular PKC activity but caused a delayed time-dependent increase in total PKA activity and synthesis. OKA suppressed the activity of the MAP kinases, ERK1/2 in a time-dependent fashion, suggesting that the Raf-1/MEKK1/MEK1/ERK1,2 cascade was compromised by OKA treatment. By contrast, OKA caused a dramatic increase in SAPK/JNK expression and activity, indicative of an activation of MEKK1/JNKK/SAPK/JNK pathway. OKA stimulated a dose-dependent activation of CAT activity using transfected promoter-CAT constructs harboring the regulatory elements AP-1 (c-*jun* promoter) and CRE (CRE-tkCAT). We conclude

Contract grant sponsors: Canadian Arthritis Society; Medical Research Council of Canada.

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Received 6 November 1997; Accepted 18 December 1997

Cellular activation by proinflammatory stimuli results in, among other responses, increased prostaglandin synthesis, which may be important in the etiopathogenesis of many immune and inflammatory diseases [Robinson et al., 1975; Dayer et al., 1976; Mizel et al., 1986; Crofford et al., 1994].Acting locally in a para-

that in primary phenotypically stable human chondrocytes, COX-2 gene expression may be controlled by critical phosphatases that interact with phosphorylation dependent (e.g., MAP kinases:AP-1, PKA:CREB/ATF) signaling pathways. AP-1 and CREB/ATF families of transcription factors may be important substrates for PP-1/PP-2A in human chondrocytes. J. Cell. Biochem. 69:392–413, 1998.

Key words: chondrocytes; cyclooxygenase-2; c-*Jun* N-terminal kinase; protein kinase A; cAMP response element

crine or autocrine fashion, prostaglandin E_2 (PGE_2) can initiate and modulate cell and tissue responses involved in many physiological processes affecting essentially all organ systems [Wu, 1996; Samuelsson et al., 1978]. The rate-limiting step in the formation of prostanoids is the conversion of phospholipid-derived arachidonic acid to PGH_2 (which is rapidly converted to PGE_2) by cyclooxygenase (COX) [DeWitt, 1991; Picot et al., 1994]. Two forms of COX have been identified: a constitutive COX-1 and the inducible COX-2 [Kubuju et al., 1991; O'Banion et al., 1992; Appleby et al., 1994]. They are integral, monotopic, endoplasmic reticulum associated, homodimeric enzymes that possess heme-dependent peroxidase and cyclooxygenase activity [Picot et al., 1994].

The COX-2 gene (mRNA 4.1 kb) is rapidly induced by tumor promoters, growth factors, cytokines, and mitogens in many cell model systems [Crofford et al., 1994; Kubuju et al., 1991; O'Banion et al., 1992]. Regulation of COX-2 expression has been shown to occur at both transcriptional and post-transcriptional levels. The COX-2 message has an extensive 3′ UTR having at least two distinct polyadenylation sites and 22 Shaw-Kamen 5′-$AUUU_n$-A-3′ motifs [Appleby et al., 1994]. The latter sequences are believed to be associated with message instability and rapid turnover [Beelman and Parker, 1995]. Sequence analysis of the 5′-flanking region has shown several potential transcription regulatory sequences, including a TATA box, a C/EBP motif, two AP-2 sites, 3 SP-1 sites, two NF-κB sites, a CRE motif, and an Ets-1 site (no AP-1 site) [Appleby et al., 1994]. Despite this wealth of structural information, it is unclear how the COX-2 gene is regulated by external stimuli in terms of signaling pathways. Recently, Inoue et al. [1995] showed that induction of COX-2 expression in vascular endothelial cells by phorbol ester or lipo-polysaccharide (LPS) involved the COX-2 CRE and NF-IL-6 sites, and that C/EBP δ functioned as a transacting factor, perhaps in association with CREB. Yamamoto et al. [1995] suggested that NF-κB and NF-IL-6, through binding to their cognate response elements, mediate COX-2 induction by tumor necrosis factor-α (TNF-α) in MC3T3E1 cells. The results reported by Xie and Herschman [1995] indicated that the signal pathway leading to *src*-induced COX-2 transcription involves both Ras/MEKK-1/JNK and Ras/Raf-1/ERK pathways.

Post-translational modification of proteins by phosphorylation plays an important role in the regulation of many cellular processes, including gene expression [reviewed by Hunter and Karin, 1992; Hunter, 1995]. Considerable evidence accrued over the years has implicated phosphorylation reactions as the molecular basis for a large number of intracellular signaling cascades. Biological processes that depend on reversible phosphorylation require not only protein kinases but also protein phosphatases and the cellular concentration of serine/threonine-directed kinases (PSTK) (but not tyrosine-specific kinases) is approximately equal to that of serine/threonine-directed phosphatases (PSTP) [Hunter, 1995]. Therefore, targeted substrate proteins are specifically phosphorylated at cognate sites by protein kinases and dephosphorylated by substrate-specific phosphatases. This being the case, modulation of kinase and/or phosphatase activity by extracellular stimuli or by pharmacological means could, in principle, alter the degree of phosphorylation at a given cognate site. In this regard, certain toxins, such as okadaic acid (OKA), a nonphorbol ester tumor promoter and an inhibitor of PSTP (i.e., PP-1 and PP-2A), have proved valuable tools in the study of cell signaling and cell cycle regulation [Alberts et al., 1993; Thévenin et al., 1990; Schönthal et al., 1991; reviewed by Schönthal, 1992]. Protein kinases, whose catalytic activities are regulated by reversible phosphorylation, have been shown to be stimulated in the presence of OKA, and it is likely that the balance of reversible cellular phosphorylation is shifted toward increased phosphorylation [Schönthal, 1992]. Unlike other tumor promoters, OKA does not directly stimulate protein kinase

C (PKC) but blocks dephosphorylation of many of its substrates. OKA induces the expression of a number of oncogenes, including members of the JUN and FOS families. As such AP-1 DNA binding activity is greatly enhanced in a variety of cell types treated with OKA as is NF-κB [Alberts et al., 1993; Thévenin et al., 1990, 1991]. OKA can transactivate promoter activity through an AP-1 site (905) and genes with promoter regions harboring these sites are dramatically induced; as for example, collagenase (MMP-1) [Westermarck et al., 1994].

Under exploratory conditions, we observed that low concentrations of OKA caused a massive increase in COX-2 gene transcription (run-off assay), steady-state mRNA expression, and protein synthesis in terminally differentiated, phenotypically stable human articular chondrocytes [Zhao et al., 1996]. This rather conspicuous "palpation" suggested to us that the signaling apparatus associated with COX-2 gene may also be equally conspicuous and quantifiable. Our data suggest that in primary, phenotypically stable human chondrocytes, COX-2 gene expression may be controlled by critical phosphatases that interact with phosphorylation dependent (e.g., MAP kinases: AP-1, PKA: CREB/ATF) signaling pathways. AP-1 and CREB/ATF families of transcription factors may be important substrates for PP-1/PP-2A in human chondrocytes. The constitutively operational raf/MEKK/MEK/ERK-1,2 signaling pathway was suppressed by OKA indicating that it may serve to control COX-2 expression in quiescent cells.

MATERIALS AND METHODS

Experimental Procedures

Chemicals. Diethylpyrocarbonate (DEPC), polyvinylpyrrolidone (PVP), Ficoll, salmon testes DNA, ethylenediamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), sodium acetate, ethidium bromide (EtBr), pyrrolidine dithiocarbamate (PDTC), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Calphostin-C, actinomycin D, α-amanitin, okadaic acid, staurosporine, and KT-5720 were the products of Calbiochem (San Diego, CA). Bio-Rad (Mississauga, Ontario) supplied acrylamide, bis-acrylamide, ammonium persulfate, tetramethylethylenediamine (TEMED), bromphenol blue, Coomassie blue G-250, and glycerol. Dulbecco's modified Eagle's medium (DMEM), HEPES, heat-inactivated fetal calf serum (FCS), stock antibiotic/antimycotic mixture (10,000 U/ml penicillin base, 10,000 µg/ml streptomycin base, and 50 µg/ml amphotericin-B), agarose, and phenol came from GIBCO-BRL (Gaithersburg, MD). Fisher Scientific (Montreal, Quebec) provided the TRIS [tris-(hydroxymethyl)aminomethane], NaCl, $MgCl_2$, $CaCl_2$, 8-hydroxyquinoline, formamide, formaldehyde, isopropanol, acetic acid, methanol, and ethanol.

Cell culture. Normal cartilage from tibial plateaus and femoral condyles were obtained at necropsy from the knee joint of human cadavers within 12 h of death. To ensure that only normal tissue was used, cartilage specimens were examined thoroughly, both macroscopically and microscopically. Only those with neither lesions nor alterations were processed further. Human chondrocytes were released from articular cartilage by sequential enzymatic digestion as previously described [Di Battista et al., 1991]. Tissue specimens were incubated at 37°C with filter-sterilized solutions of pronase or trypsin (1 mg/ml, 1.5 h) and collagenase (1 mg/ml, 6 h) in DMEM containing 10% heat-inactivated FCS and an antibiotic mixture (100 U/ml penicillin, 100 µg/ml streptomycin, and 0.5 µg/ml amphotericin-B). Cell viability was determined by the trypan blue exclusion test. In order to maintain a terminally differentiated chondrocyte phenotype, primary cultures were seeded at high density and were used at a confluent and stationary phase (1–2 days).

Northern Blot Analysis of mRNA

Total cellular RNA was isolated (1×10^6 cells = 10–20 µg RNA) using the Trizol (GIBCO) reagent. Generally, 10–15 µg of total RNA was resolved on 0.9% agarose-formaldehyde gel and transferred to Hybond-N™ nylon membranes (Amersham, Canada Ltd., Oakville, ON) in 20 × SSC buffer, pH 7 by vacuum blotting. After prehybridization for 24 h, hybridizations were carried out at 50–57°C (depending on the gene) for 24–36 h, followed by high-stringency washing, as previously described [Di Battista et al., 1994a]. The following probes, labeled with digoxigenin (DIG)-dUTP by random priming, were used for hybridization: human COX-2 cDNA (1.8 kb) (Cayman Chemical Company, Ann Arbor, MI) was cloned into the *Eco*RV site of pcDNA 1 (Invitrogen) and released by *Pst*I and *Xho*I digestion. Mouse *jun*B and D were 1.6 and

1.0 *Eco*RI and *Acc*I/*Eco*RI cDNA fragments, respectively, from pBluescript SK^- (kindly provided by Dr. D. Skup, Louis-Charles Simard Research Center, Montreal, Canada). The mouse c-*jun* 1.8-kb cDNA probe (provided by Dr. D. Edwards, University of Calgary) was extracted from a pUC19 plasmid using *Eco*RI. The 1.3-kb cDNA mouse c-*fos* probe was originally cloned into pGEM II (Dr. J.P. Pujol, Université de Caen, France) and was excised for labeling using *Pst*I and *Bcl*I. The mouse *fos*-B 1.6 cDNA probe was kindly provided by Dr. D. Skup. All blots were subjected to laser scanning densitometry (GS-300, Hoefer Scientific Instruments, San Francisco, CA) for semiquantitative measurements, with the relative amount of test mRNA normalized to the level of 28S/18S rRNA (negative image of EtBr staining pattern of membrane). OKA down-regulates the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), actin, and tubulin mRNA in a time-dependent fashion and, as such, they are ill-suited as controls. The expression is stable for up to 2 h but declines to 15–20% of the control values after 20 h (data not shown).

Isolation of nuclei and transcriptional runoff assay. Nuclei from treated and control cells were isolated by high-speed centrifugation through 1.9 M sucrose-density gradient as previously described [Di Battista et al., 1994b]. The nuclei were then resuspended in a buffer of 50 mM Tris–HCl, pH 8.0, 5.0 mM magnesium sulfate, 0.1 mM EDTA, 1.0 mM DTT, and 25% glycerol. Nuclei were snap-frozen in liquid nitrogen and stored in the same until used. Approximately 20×10^6 nuclei were used per transcription reaction, which consisted of mixtures (final volume, 200 µl) containing 25 mM Tris (pH 8.0), 12.5% glycerol, 120 mM KCl, 10 mM $MgCl_2$, 2.5 mM DTT, and 2 mM each of ATP, CTP, GTP, 5 µM ^{32}P-UTP (100 µCi). RNA synthesis was allowed to proceed for a maximum of 30–60 min at 25°C, with regular monitoring of the elongation rate in both treated and control samples. RNA polymerase II activity was verified with the addition of 100 ng/ml of α-amanitin and 2 µg/ml of actinomycin D. Reactions were terminated by adding a solution of 1 mM EDTA, 10% SDS, (pH 7.0), resulting in a complete solubilization of the nuclei. Labeled RNA was isolated as described above.

To specifically quantitate COX-2 labeled mRNA, linearized heat- and base-denatured COX-2 cDNA containing plasmid (5 µg) was spotted onto nitrocellulose membranes (Bio-Rad, Richmond, CA) with the aid of a 96-well manifold apparatus (Bio-Rad). Membranes were prehybridized, then hybridized with approximately 100,000 dpm of labeled RNA transcripts derived from control and treated nuclei as per Northern blotting procedures. Signal intensity was measured by laser scanning densitometry after appropriate background subtraction obtained using linearized pcDNA 1. A 780-bp PstI/XbaI fragment from GAPDH cDNA (1.2 kb; American Type Culture Collection, Rockville, MD) served as a control as GAPDH is constitutively expressed and the transcriptional rate should be relatively constant.

Preparation of cell extracts and Western blotting. A total of 50–100 µg of cellular extract (in RIPA buffer; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 µg/ml each of aprotinin, leupeptin, and pepstatin, 1% NP-40, 1 mM sodium orthovanadate, and 1 mM NaF) from control and treated cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through 10–12% gels (final concentration of acrylamide) under reducing conditions, and transferred onto nitrocellulose membranes (Amersham). After blocking (with 5% BLOTTO) and washing, the membranes were incubated for either 2 h at RT, or overnight at 4°C, with primary antibodies (see below) in TTBS containing 0.25% BLOTTO. Second anti-rabbit or anti-mouse antibody-HRP conjugates (1:2,000 dilutions) were subsequently incubated with membranes for 1 h at room temperature (RT) and then washed extensively for 30–40 min with TTBS, and a final rinsing with TBS at RT. After incubation with an ECL chemiluminescence reagent (Amersham), membranes were prepared for autoradiography and exposed to Kodak X-Omat film, then subjected to laser scanning densitometry for semiquantitative analysis. The antibodies used were a polyclonal anti-human COX-2 (Cayman Chemical Co., 1: 5,000 dilution); Fra-1, CREB-1/CREB-1-P, and ATF-1/ATF-1-P which were products of Santa Cruz Biotechnology (Santa Cruz, CA) and New England Biolabs (Beverly, MA), respectively, and used at dilutions suggested by the manufacturer.

Gel-retardation experiments. Confluent chondrocytes in 4-well cluster plates ($3–5 \times 10^6$ cells/well) were treated with OKA (30 nM) for 0–20 h, after which the cells were carefully

scraped into 1.5 ml ice-cold PBS and pelleted by brief centrifugation as previously described [Ney et al., 1990]. The cellular pellet was gently resuspended in 200–400 µl ice-cold hypotonic lysis buffer containing 10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM Pefabloc™, 10 µg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM sodium orthovanadate, 1 mM NaF and 1% Nonidet P-40. Cells were allowed to swell on ice for 10 min, vortexed vigorously for 10 s, and nuclei recovered by brief centrifugation at 3,000*g* for 60 s. The nuclear pellets were resuspended in 25 µl of high salt extraction buffer containing 20 mM HEPES–KOH, pH 7.9, 0.42 M NaCl, 1.2 mM $MgCl_2$, 0.5 mM DTT, 0.3 mM EDTA, 25% glycerol, 0.5 mM Pefabloc™, and 10 µg/ml each of aprotinin, leupeptin and pepstatin, followed by incubation on ice for 45 min with intermittent vortexing. The nuclear extracts were recovered by centrifugation at 16,000*g* for 30 min at 4°C and stored at −86°C until used.

Double-stranded oligonucleotides containing consensus and promoter-specific sequences, synthesized, annealed, and purified by high-performance liquid chromatography (HPLC), were end-labeled with [γ-^{32}]ATP using T4 polynucleotide kinase (Pharmacia, Montreal, Quebec). The sense sequences of the oligos used were as follows: AP-1; 5′-CGC TTG ATG AGT CAG CCG GAA-3′: NF-κB; 5′-AGT TGA GGG GAC TTT CCC AGG C-3′: CRE; 5′-AGA GAT TGC C TG ACG TCA GAG AGC TAG-3′: CRE-COX-2; 5′-AGA GAT TGC CTT TCG TCA GAG AGC TAG-3′: SP-1; 5′-ATT CGA TCG GGG CGG GGC GAG C-3′: AP2; 5′-GAT CGA ACT GAC CGC CCG CGG CCC GT-3′: NF-IL-6-COX-2; 5′-CAC CGG GCT TAC GCA ATT TTT TTA A-3′. Binding buffer consisted of 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol and 2.5 µg poly(dI-dC). Binding reactions were conducted with 15 µg nuclear extract and 100,000 cpm of ^{32}P-labeled oligonucleotide probe at 22°C for 20 min in a final volume of 10 µl. In "supershift" studies, 1 µg antibody (e.g., anti-JUN B) was added to the reaction mixture and incubated for another 45 min at 22°C. Binding complexes were resolved by nondenaturing PAGE through 6% gels in a Tris-borate buffer system, after which the gels were fixed, dried and prepared for autoradiography.

Analysis of protein kinase activity. Chondrocytes were incubated with OKA (30 nM) for 0–20 h and then rinsed with ice-cold PBS and scraped from the plates, pelleted and extracted into RIPA buffer 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 µg/ml each of aprotinin, leupeptin, and pepstatin, 1% NP-40, 1 mM Na_3VO_4, and 1 mM NaF. For the phosphatase assays, Na_3VO_4 and NaF were omitted. Generally, 20–40 µg protein was used per assay (linear range 0–100 µg).

For protein kinases A and C (PKA and PKC) nonradioisotopic techniques were employed, based on an enzyme-linked immunosorbent assay (ELISA) (Upstate Biotechnology, Lake Placid, NY). The procedure uses a synthetic peptide as kinase substrate and a monoclonal antibody (MAb) (2B9) that recognizes the phosphorylated form of the peptide (R-F-A-R-K-G-pS-L-R-Q-K-N-V). The PKA and PKC present in samples phosphorylate the peptide (recognition motif underlined) coated on microwell plates, and a biotinylated MAb, 2B9, binds the phosphorylated peptide and is detected with streptavidin conjugated to peroxidase. Peroxidase substrate is added to the microwells, and the color intensity is measured spectrophotometrically at 490 nm. Standard curves were generated with increasing concentrations of purified PKC (0–100 ng: rat brain, mixture isozymes α, β, γ) (Upstate Biotechnology) and the catalytic subunit of PKA (0–500 ng: bovine heart) (Upstate Biotechnology). Results are expressed as ng equivalents per 100 µg of cellular protein; the change in optical density obtained with a fixed amount of cellular extract corresponded to an equivalent change in optical density for a given amount of purified enzyme. Specificity was controlled using specific inhibitors of PKA (PK inhibitor peptide—TYADF-IASGRTGRRNAI) (Upstate Biotechnology) and KT-5720, 2 µM, PKC (PKC inhibitor peptide corresponding to amino acids 19–31 (RFA-RKGALRQKNV) of PKC and termed a "pseudo-substrate," since it functions as an endogenous auto-inhibitor of PKC activity, and calphostin C, 100 nM, and calcium/calmodulin dependent kinase (R24571). As this last procedure, although offering a number of advantages, is a rather recent technological development, we verified our results using a classic procedure involving the ^{32}P-phosphorylation ([γ-^{32}P]-ATP) of specific substrates for PKC (QKRPSQRS-KYL) and PKA (so-called Kemptide, LRRASLG-OH) in the presence (total activity) or absence (activated) of (1) Ca^{2+}, phosphatidylserine, dia-

cylglycerol, and Mg^{2+} ions for PKC, or (2) cAMP for PKA. Incorporated radioactive phosphate was measured by spotting reaction mixtures on P81 phosphocellulose filter disks, washing with phosphoric acid, and scintillation counting. Reaction specificity was controlled with specific chemical inhibitors as in the nonradioisotopic procedure.

The mitogen-activated protein (MAP) kinase and the stress-activated protein kinase (SAPK/JNK) assays were also measured by nonradioisotopic procedures (New England Biolabs, Beverly, MA) by taking advantage of phospho-specific antibodies. In the former case, a phospho-specific antibody to MAP-kinases (Tyr204) were used to immunoprecipitate active MAP kinases (p42/44, ERK-1, and ERK-2) selectively from chondrocyte lysates and the latter immunoprecipitates were then incubated with an Elk-1 (substrate) fusion protein in the presence of ATP and kinase buffer. Phosphorylation of Elk-1 at Ser383 was measured by Western blotting using a phospho-specific Elk-1 (Ser383) antibody. Ser383 of Elk-1 is a major phosphorylation site for MAP kinase and is required for Elk-1-dependent transcriptional activity [Karin, 1995; Cobb and Goldsmith, 1995]. For SAPK/JNK assays, an N-terminal c-JUN (1-89)-GST fusion protein bound to glutathione Sepharose beads was used to selectively "pull-down" SAPK/JNK from chondrocytes lysates. The fusion protein is known to have a high-affinity site for SAPK/JNK binding [Kallunki et al., 1996]. The beads were then mixed with ATP and kinase buffer and c-JUN phosphorylation was selectively measured using phospho-specific c-JUN antibody. The latter antibody specifically detects SAPK-induced phosphorylation of c-JUN at Ser63, a site important for c-JUN-dependent transcriptional activity [Kallunki et al., 1996].

Serine/threonine protein phosphatase assays were performed by monitoring the dephosphorylation of a PKC-phosphorylated peptide R-F-A-R-K-G-pS-L-R-Q-K-N-V previously coated on microwell plates. The biotinylated MAb, 2B9, binds the phosphorylated peptide and is detected with streptavidin conjugated to peroxidase. Peroxidase substrate is added to the microwells and the decrease in optical density is measured spectrophotometrically at 490 nm with increasing protein phosphatase activity. A standard curve analysis using purified protein phosphatase-1 (0–100 ng) (Upstate Biotechnology), was performed to estimate endogenous chondrocyte serine/threonine protein phosphatase activity.

Cell transfections and reporter assays. Transient transfection experiments were conducted in 60-mm culture dishes with 5×10^5 cells that were serum starved for 24 h before use. Transfections were conducted by the calcium phosphate/DNA coprecipitation method followed by glycerol shock for 45 s. Cells were re-exposed to a complete culture medium for 16 h before the addition of increasing concentrations of OKA (0–60 nM) for an additional 20 h. Transfection efficiencies were controlled by cotransfection with 0.5 μg of pRSV-βgal, a β-galactosidase reporter vector under the control of RSV-LTR promoter. The following promoter constructs were used: c-*jun* promoter: pBLCAT3 vectors with −79/+170 jun-CAT and −79/+170 ΔAP-1 CAT where the wt AP-1 consensus sequence is mutated (a gift from Dr. M. Karin). pCRECAT2 (4xtkCAT), which contains four CRE consensus in front of the tk promoter in the pBLCAT2 plasmid. CAT assays were measured by the scintillation technique [Neumann et al., 1987] and verified using specific CAT ELISA (Boehringer-Mannheim, Canada). CAT values were normalized to the level of β-galactosidase activity.

Radioimmunoassay of PGE_2 in culture medium. PGE_2 in control and treated culture medium will be quantitated by the PGE_2 [^{125}I] scintillation proximity assay (Amersham) after methyloximation of PGE_2.

Statistical analysis. All results are expressed as mean ± standard deviation of between two to five separate experiments. Statistical significance will be assessed using the Student's t-test and significant differences were confirmed only when the probability was less than or equal to 5%.

RESULTS

Okadaic Acid Causes a Time- and Dose-Dependent Increase in COX-2 Synthesis and mRNA Expression by Transcriptional Means

With high-density primary cultures of human articular chondrocytes, OKA (30 nM) stimulated an increase in the steady-state levels of 4.1 kb COX-2 mRNA in a delayed fashion requiring up to 4 h to obtain a response as judged by Northern blotting using a specific human COX-2 cDNA probe (Fig. 1). Levels reached a zenith at 20–24 h (fold induction 10.7 ± 2.6 normalized to 28 S and 18S rRNA,

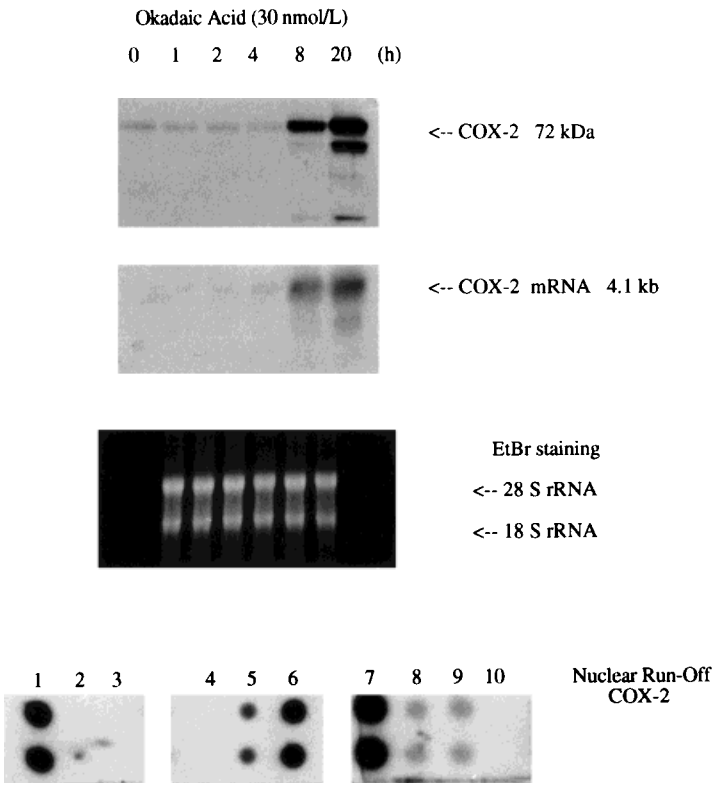

Fig. 1. Time course of OKA-induced up-regulation of COX-2 gene transcription, mRNA, and protein in human articular chondrocytes. Normal chondrocytes were seeded at high density (1.2×10^6 cells per well) and confluence (stationary phase) was reached in 1–3 days. The cells were then serum starved for 24 h and then incubated with 30 nmol/L OKA for the indicated times. Cellular proteins were extracted into RIPA buffer and analyzed by Western blotting, using a specific anti-COX-2 antiserum. RNA was extracted using the TRIZOL reagent and analyzed by Northern blotting using specific probes for COX-2. Nuclei (20×10^6 in T150 flasks) were isolated at the appropriate times and runoff assays (in duplicate) were performed as described under Materials and Methods. **Lane 1,** control at time 0 h, transcripts hybridized to a GAPDH probe; **lanes 2–7,** time course OKA stimulation 0, 1, 2, 4, 8, 20 h labeled transcripts hybridized to a COX-2 probe; **lanes 8 and 9,** 20 h OKA-stimulated runoffs in the presence of α-amanitin (100 ng/ml) and actinomycin D (2 µg/ml) and hybridized to a COX-2 probe; **lane 10,** 20 h OKA-stimulated runoffs hybridized to a pcDNA1 plasmid (minus COX-2 insert).

n = 5) and declined thereafter (data not shown). The latter decline was probably due to cell toxicity that we observed with prolonged incubations. Nuclear run-off experiments suggested that this induction was due to an increase in the rate of COX-2 gene transcription as the levels of nascent transcripts coincided with steady-state COX-2 mRNA levels assayed by Northern blotting (Fig. 1). Transcriptional increases were abolished in the presence of 100 ng/ml α-amanatin and 2 µg/ml of actinomycin D, both inhibitors of RNA polymerase II activity in eukaryotic cells. Changes in the synthesis of COX-2 were apparent sometime after 4 h as we first detected the 70- to 72-kDa enzyme doublet (differential glycosylation) at 8 h (Fig. 1). The synthesis of COX-2 reached a maximum after 20–24 h (fold induction 11.4 ± 1.7, n = 5) after which time the levels declined (data not shown). With some specimens, we observed >50 fold increases in COX-2 after 20–24 h of exposure to OKA. Bands appearing at 50–60 and 25–30 kDa are degradation products and have been identified previously [Sirois and Richards, 1992].

When chondrocytes were treated with increasing concentrations of OKA (0–120 nM) for 20 h, a maximum induction was attained when the concentration reached 15–30 nM but declined at higher concentrations perhaps reflecting an increase in cellular toxicity (Fig. 2). To test the dependency of OKA-induced COX-2 mRNA expression on new protein synthesis, 1 and 10 µg/ml cycloheximide was co-incubated with OKA and found to cause a small super induction (1.8 ± 0.2 fold) of COX-2 mRNA at 10 µg/ml. The pattern of COX-2 synthesis, as a function of the concentration of OKA, mirrored the COX-2 mRNA levels (Fig. 2). Calyculin A, also an inhibitor of PP-2A and PP-1 that is 10-

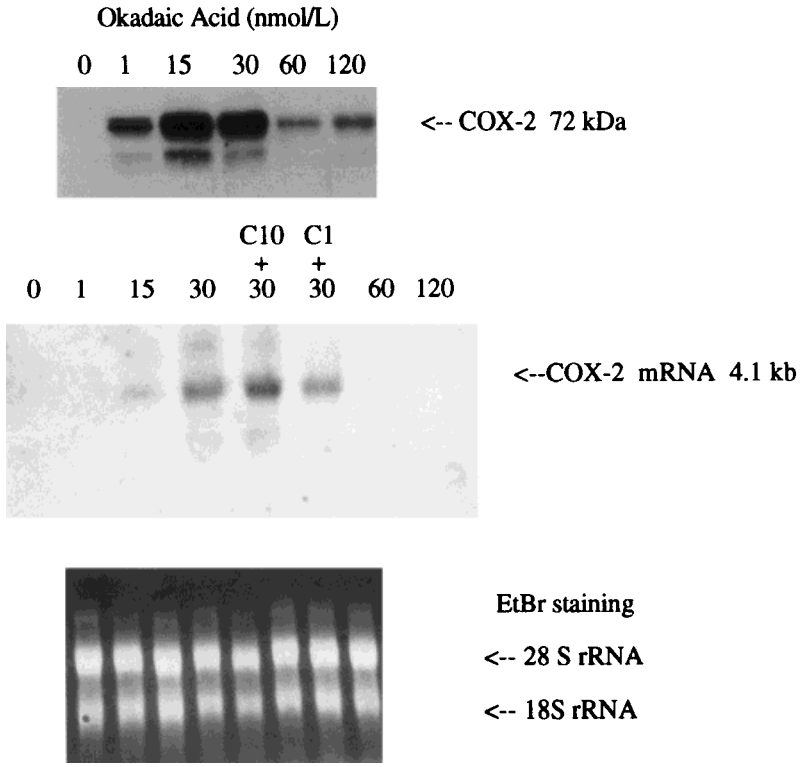

Fig. 2. Dose response of OKA-induced up-regulation of COX-2 protein and mRNA expression. Effect of cycloheximide. Serum-starved, confluent quiescent chondrocytes were incubated with increasing concentrations of OKA (0–120 nmol/L) for 20 h as indicated. Cellular proteins were extracted into RIPA buffer and analyzed by Western blotting using a specific anti-COX-2 antiserum. RNA was extracted using the TRIZOL reagent and analyzed by Northern blotting, using a specific probe for COX-2. C10, 10 µg/ml cycloheximide, co-incubated with 30 nmol/L OKA; C1, 1 µg/ml cycloheximide, co-incubated with 30 nmol/L OKA.

to 20-fold more potent than OKA, stimulated COX-2 synthesis with an EC_{50} of 0.5 nM and the maximum induction was reached at 1.5 nM (data not shown).

Okadaic Acid Causes a Time- and Dose-Dependent Increase in Oncogene/Transcription Factor mRNA Expression and Synthesis

Northern analysis of total RNA extracts from chondrocytes treated with 30 nM of OKA for 0–20 h showed that c-*jun*, *jun*B, *jun*D, and c-*fos* (Fig. 3, rows 1–4), were all up-regulated by the treatment. However, c-*fos* and c-*jun* transcripts were not detectable in resting cells, while *jun*B, and especially *jun*D, exhibited significant basal expression. Induction of c-*fos* and c-*jun* required 2–4 h, reaching a maximum at 8 h, the same amount of time required for peak up-regulation of *jun*B and *jun*D. In contrast to the other proto-oncogenes, *jun*D was expressed at fairly high levels in quiescent chondrocytes and as such the fold induction was lower than the others (cf. 2.3 ± 0.4 vs 4.5 ± 0.9, 7.1 ± 2.1, 9.6 ± 3.3 for *jun*B, c-*jun*, and c-*fos*, respectively, $n = 3$). Fra-1 was unaffected by OKA, and this was confirmed at the protein level by Western analysis of RIPA extracts using a anti-*Fra*-1 antibody (Fig. 3, row 5). Fos B mRNA was not detectable in human chondrocytes following OKA treatment (data not shown).

Previous studies suggest that OKA has no effect on CRE modulated gene expression in NIH 3T3 cells, although it could augment the level of phosphorylation of CREB-1 in forskolin-treated PC12 cells [Schöntal et al., 1991; Hagiwara et al., 1992], and the latter transcription factor may be a substrate for PP1 and PP2A [Hagiwara et al., 1992]. We examined this possibility in chondrocytes treated with 30 nmol/L of OKA for 0–24 h and found that, indeed, the level of phospho-CREB-1 (S133) was significantly increased beginning at 4 h and reaching a maximal induction at 8 h, declining thereafter (Fig. 4, top). A faster moving protein species was also detected and represents ATF-1 with

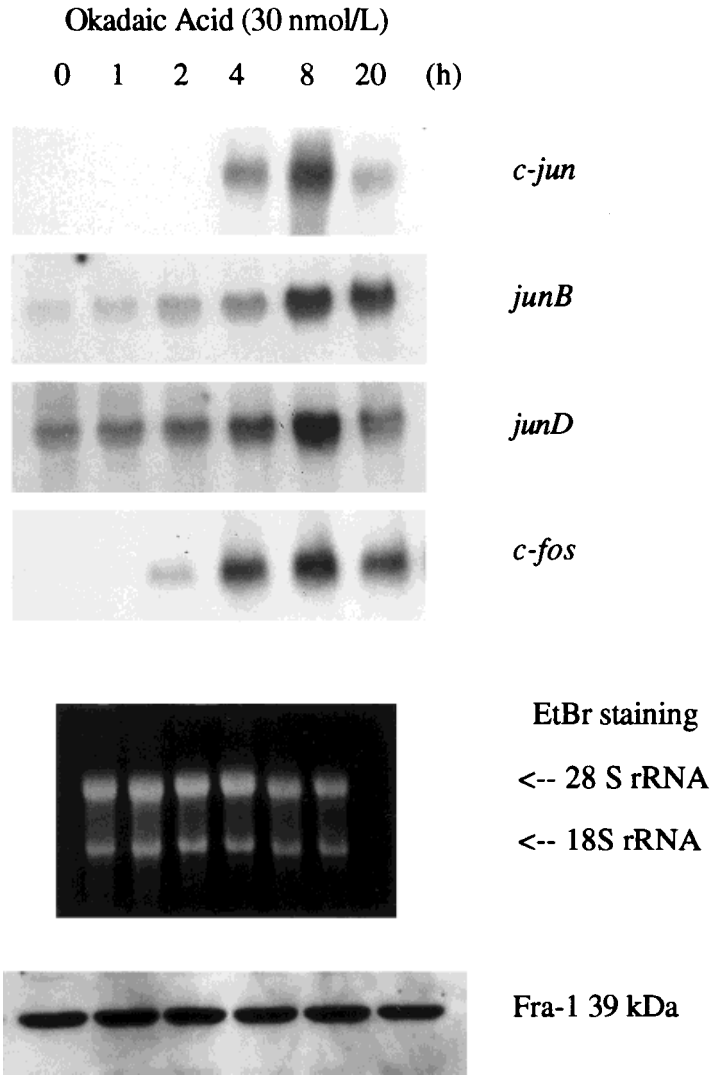

Fig. 3. Time course of OKA induction of members of the c-*jun* and c-*fos* families of proto-oncogenes. Serum-starved, confluent quiescent chondrocytes were incubated with 30 nmol/L OKA for 0–20 h as indicated. RNA was extracted using the TRIZOL reagent and analyzed by Northern blotting, using specific probes for c-*jun,* *jun*B, *jun*D, c-*fos,* and *fos*B (not shown). The ethidium bromide staining is representative of multiple blots used to assess mRNA levels for the protooncogenes. In some experiments, cellular proteins were extracted into RIPA buffer and analyzed by Western blotting, using a specific anti-Fra-1 monoclonal antibody.

which the antibody cross-reacts. Total CREB-1 (nonphosphorylated/phosphorylated) levels appeared to be quite high and unchanged during the incubation period (Fig. 4, bottom).

Okadaic Acid Stimulates AP-1 and CREB DNA Binding Activity

Mobility gel shifts were performed to determine whether the increase in AP-1 and CREB expression and synthesis following OKA treatment would be reflected in an increase in binding to cognate DNA regulatory element sequences. Indeed, we observed a dramatic increase in AP-1 binding to a ^{32}P-labeled AP-1 consensus sequence in a time-dependent fashion with a maximum being reached after 18–20 h (Fig. 5, top). A similar pattern was also obtained with a ^{32}P-labeled CRE sequence, although the level of binding was somewhat lower (23% of AP-1 binding, mean of three determinations) (Fig. 5, middle); both ^{32}P-labeled oligonucleotides had almost identical specific activities, about ±5% of one another. A small but inconsistent increase in NK-κB binding was observed and represents only about 5% of what one obtains when chondrocytes are activated

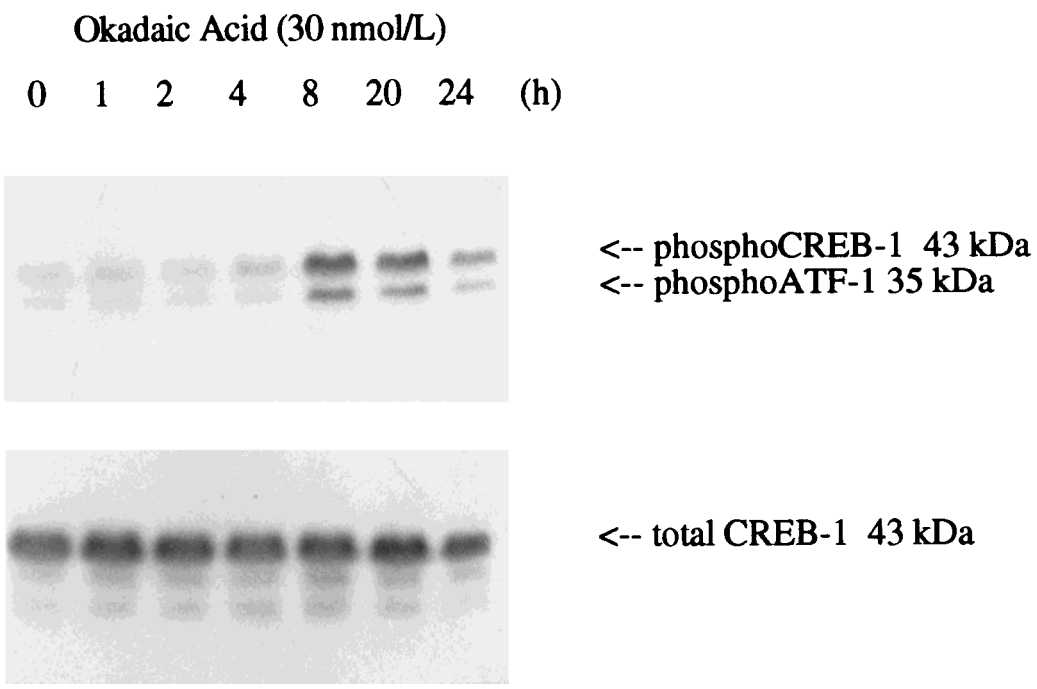

Fig. 4. Time course of OKA induced phosphorylation of CREB-1/ATF-1 transcription factors. Serum-starved, confluent quiescent chondrocytes were treated with 30 nmol/L OKA for 0–24 h as indicated. Cells were washed with ice-cold PBS and then solubilized in 100 µl boiling SDS sample buffer. A total of 100 µg of the whole cell extracts was resolved by PAGE and subjected to Western blotting, using specific antiserum to total CREB-1 and phosphoCREB-1. The antibody has been shown to cross-react only with ATF-1.

with TNF-α (Fig. 5, bottom left and right). We were unable to detect increases in AP-2, Oct-1, SP-1, or NF-IL-6-COX-2 binding activity although all the latter cognate consensus sequences can be found in the promoter region of the COX-2 gene [Appleby et al., 1994; Inoue et al., 1995].

There is evidence that phosphorylation by protein kinase A plays a crucial role in terms of DNA binding and promoter trans-activation of CREB/ATF family members and we sought to determine whether OKA stimulation of CRE binding in chondrocytes stimulated a similar signalling apparatus. To this end, we used KT-5720 (2 µM), a fairly specific PKA inhibitor, and found that the DNA binding capacity of OKA-treated chondrocyte nuclear extracts to labeled CRE consensus oligonucleotides was abrogated (Fig. 6, lane 3). To test the specificity of the kinase response, we repeated the experiments with an inhibitor of PKC, calphostin C, and found either no effect or a slight elevation in the binding (Fig. 6, lane 4). Binding was displaced by COX-2 CRE at one-fold molar excess cold oligonucleotide in a manner identical to the consensus CRE (see Fig. 5, top).

Studies have shown that AP-1/c/EBP family members can dimerize with CREB/ATF monomers and as such greatly increase the diversity and specificity of cellular responses to a variety of extracellular signals [Ryseck and Bravo, 1991]. Along these lines, we wondered whether CRE binding proteins from OKA-treated chondrocyte extracts could also interact with AP-1 or NF-IL-6 sequences and found only marginal displacement under our experimental conditions (Fig. 7, lanes 3 and 4). Parenthetically, these findings support our previous observations (not shown) with respect to direct binding of chondrocyte extracts to NF-IL-6–COX-2 sequences. Interestingly, preincubation of OKA-treated nuclear extracts with an antibody to the CREB binding protein (CBP), a coactivator of cAMP inducible genes [Kamei et al., 1996], abolished CRE binding (Fig. 7, lane 5).

PKA and PKC would not appear to affect significantly the binding of OKA-treated chondrocyte nuclear extracts to AP-1 judging by the gel-shift results in the presence of KT-5720 and calphostin C (Fig. 8, lanes 3 and 4). In fact, calphostin C either had no effect or caused a mild up-regulation (depending on the speci-

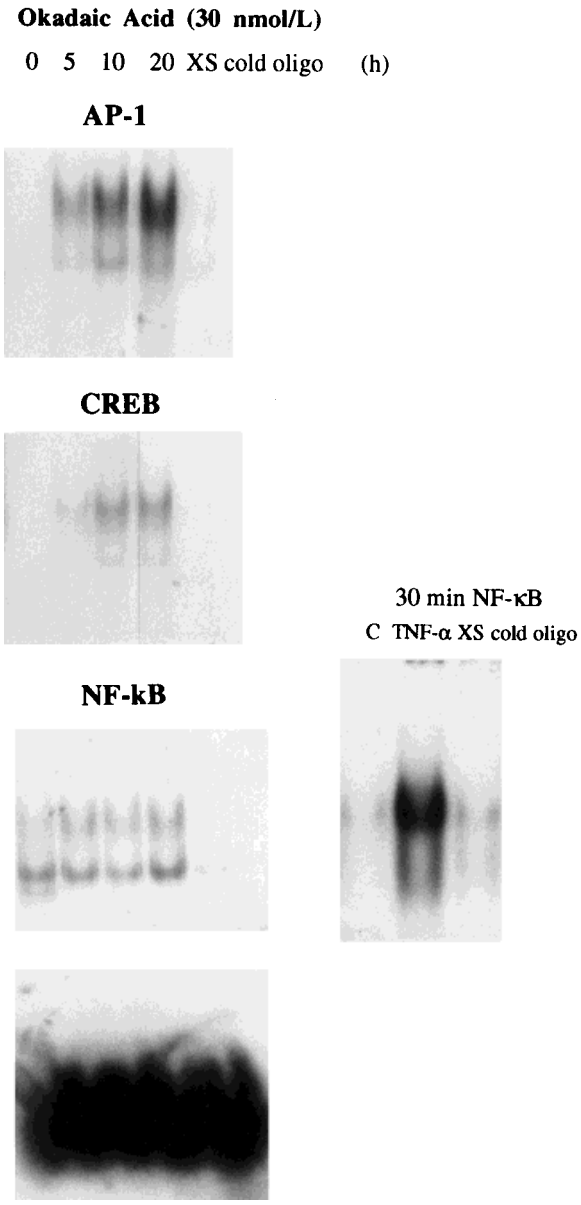

Fig. 5. Time course of OKA induction of AP-1, CRE, and NF-κB consensus sequence nuclear binding activity. Normal chondrocytes were seeded at high density, and confluence was reached in 1–3 days. The cells were then serum starved for 24 h and then incubated with 30 nmol/L OKA for 0, 5, 10, and 20 h. Nuclei were isolated by hypotonic lysis of the chondrocytes and proteins extracted into high salt buffer as described under Materials and Methods. A total of 15 μg nuclear protein was incubated with ^{32}P-labeled AP-1, CREB, and NF-κB consensus sequences in the absence or presence (20-h extracts only) of a one-fold molar excess of radioinert homologous oligonucleotide (lane 5 of each gel). Mobility shifts were verified on 6% polyacrylamide gels followed by gel drying and autoradiography. NF-κB induction with 5 ng/ml TNF-α for 30 min is illustrated to show that OKA is not an efficient NF-κB inducer in human chondrocytes. Bottom, representation of free probe.

men) in the binding to AP-1 consensus oligonucleotides. As expected, a one-fold excess of AP-1 oligo displaced virtually 100% of the binding but, interestingly, a COX-2 CRE consensus oligo also displaced 80–90% of the binding as well (Fig. 8, lanes 5 and 6). In order to further elucidate the composition of these AP-1 binding proteins, we performed super-gel-shift analysis (see above) with antibodies to c-Jun, c-Fos, c-JunD, and c-JunB. Only anti-c-Fos and JunB antibodies shifted a small percentage of the complexes while anti c-Jun antibodies always increased the binding by 20–30%; anti-JunD antibodies had no discernible effect (Fig. 9, lanes 3–6).

Okadaic Acid Stimulates PKA and SAPK/JNK Activity But Not PKC or MAP Kinase (ERK-1/2): Time Line of Serine/Threonine Phosphatase Inhibition by OKA

Given the up-regulation in terms of the expression and DNA-binding capacity of AP-1 and CRE binding macromolecular species and the fact that their functionality depends on targeted phosphorylation by specific kinases, we assayed for kinase activity associated with the phosphorylation of these two transcription factor families. Basal levels of PKA activity are relatively low in quiescent chondrocytes, as are intracellular levels of cAMP because of the high activity of cAMP-dependent phosphodiesterase IV [Di Battista et al., 1996]. However, in the presence of 30 nmol/L of OKA total assayable cAMP-dependent PKA was stimulated in a delayed, time-dependent fashion with significant increases appearing at 4 h and reaching virtual saturation at 8 h (Fig. 10A). We detected slight increase at 20 h, but they were not significantly different from 8-h values (Fig. 10A). There was no dramatic concomitant increase in cAMP levels with the intracellular concentration remaining essentially unchanged for the duration of the incubation period (225 fmol/10^6 cells vs 184 fmol/10^6 cells at 20 h: mean of two determinations). OKA had no effect on the amount of total assayable PKC even after 20 h of stimulation, which corroborates previous results in other cell model systems [Schönthal, 1992] (Fig. 10A).

The delayed response of chondrocytes to OKA in terms of target gene expression (see above) may be attributable to inhibition of endogenous

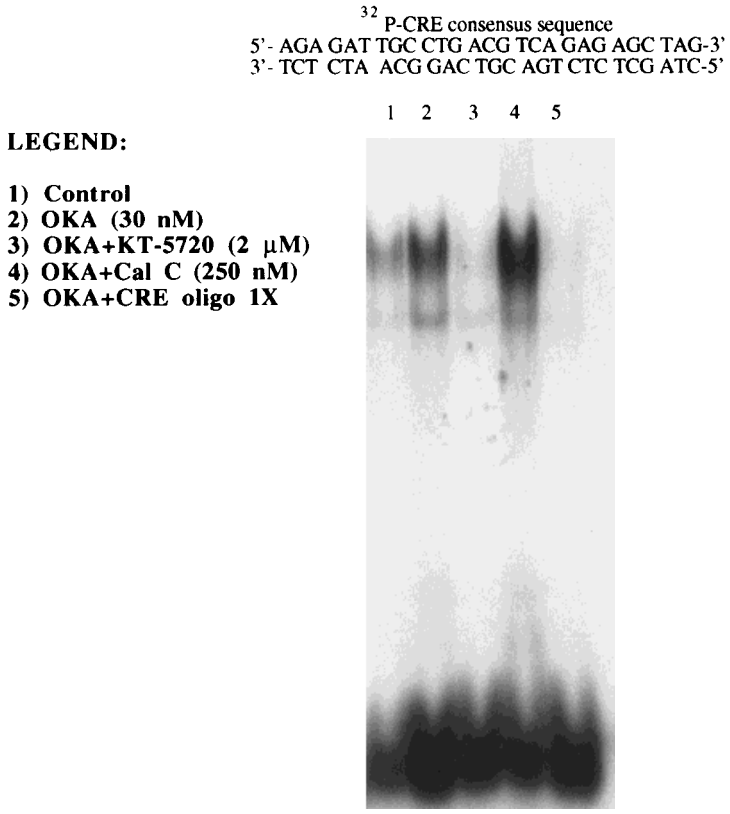

Fig. 6. OKA induction of CRE nuclear binding activity is inhibited by protein kinase A (PKA) inhibitor, KT-5720. Serum-starved, confluent quiescent chondrocytes were incubated without **(lane 1)** or with **(lane 2)** 30 nmol/L OKA in the presence of the specific PKA inhibitor KT-5720 (2 μM) **(lane 3)** or the specific PKC inhibitor calphostin C (CalC, 100 nM) **(lane 4)** for 20 h. Nuclei were isolated by hypotonic lysis of the chondrocytes and proteins extracted into high salt buffer. A total of 30 μg of protein was incubated with ^{32}P-labeled CRE consensus sequence and subjected to electrophoresis on 6% polyacrylamide gels. **Lane 5,** displacement of ^{32}P-labeled CRE consensus sequence with one-fold molar excess of COX-2 CRE.

protein phosphatase 1 and 2A requiring up to 4 h before an inhibitory affect is seen. As shown in Figure 10B, inhibition of cellular phosphatase activity began around 4 h and continued its downward trend for up to 20 h, inhibiting 89.6 ± 5.3% (n = 3) of the activity. The OKA-formed protein phosphatase noncovalent reversible complex, form intracellularly, must have been stable to our extraction procedures as judged by our assay results. These data were strongly corroborated by in vitro studies which showed that OKA will inhibit up to 96% of basal serine/threonine protein phosphatase activity in untreated chondrocyte soluble extracts (data not shown).

Given the potential role of MAP kinase cascades in the regulation of AP-1 activity [Karin, 1995], we measured the activity of the MAP kinases p42/44 (ERK1/2) in chondrocyte lysates after treatment with OKA (30 nM) for 0–20 h. Using phosphospecific antibodies (Tyr204 of MAP), active ERK1/2 were immunoprecipitated, and the phosphorylation of a specific substrate, Elk-1, was monitored by Western blotting, using a specific phospho (Ser383) antibody. Inhibition of active ERK1/2 was evident beginning at 4 h and was essentially undetectable after 20 h, as judged by the disappearance of the phosphorylated form of Elk-1 (Fig. 11, top).

Another proline-directed MAP kinase more intimately associated with c-Jun phosphorylation, c-Jun kinase (SAPK/JNK) [Kallunki et al., 1996] was also assayed after OKA treatment and the results are shown in (Fig. 11, bottom). The latter kinase phosphorylates c-JUN at ser 63 and renders it capable of dimerizing, binding DNA and transactivating target genes. OKA induced the activity of JNK in a delayed time-dependent fashion reaching a zenith after 8–10 h, although there was measureable activity after 1–2 h of OKA stimulation.

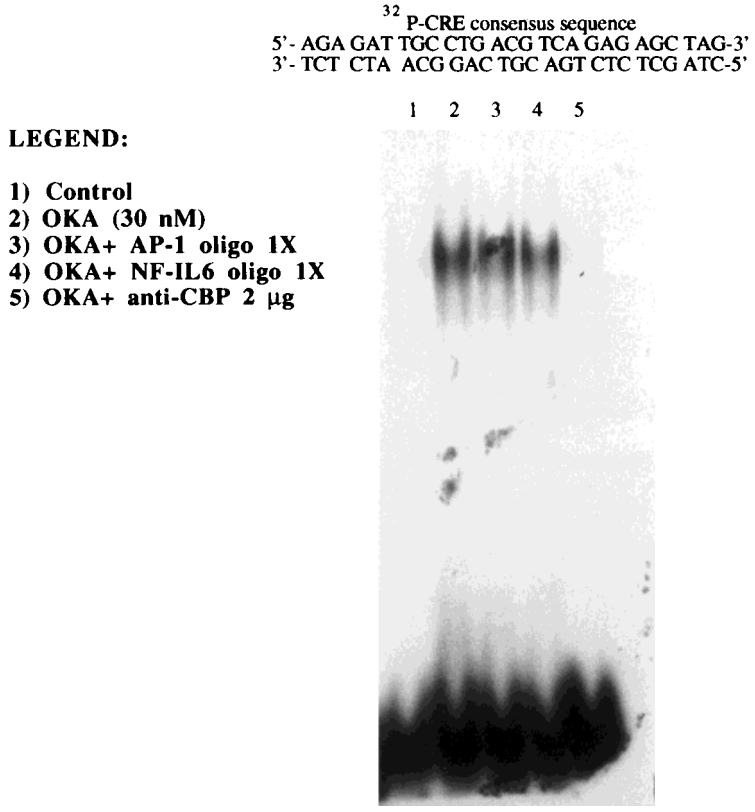

Fig. 7. COX-2-NF-IL-6 and AP-1 consensus oligo nucleotides do not compete for OKA-induced CRE nuclear binding activity. Serum-starved, confluent quiescent chondrocytes were incubated without **(lane 1)** or with **(lane 2)** 30 nmol/L OKA for 20 h. Nuclei were isolated by hypotonic lysis of the chondrocytes and proteins extracted into high salt buffer. A total of 30 μg of protein was incubated with ^{32}P-labeled CRE consensus sequence and subjected to electrophoresis on 6% polyacrylamide gels. **Lanes 3** and **4,** displacement patterns of ^{32}P-labeled CRE consensus sequence nuclear binding activity with one-fold molar excess of radioinert AP-1 or COX-2 NF-IL-6, respectively. **Lane 5,** displacement of ^{32}P-labeled CRE consensus sequence nuclear binding activity with an anti CREB-binding protein (p265) antiserum.

OKA Up-Regulates Transfected Promoter/Reporter Gene Constructs Containing CREB and AP-1 Consensus Sequences

Though OKA stimulated a large increase in CREB/ATF phosphorylation and CRE DNA binding activity, this does not necessarily mean that there will be an increase in tandem of transacting activity mediated through the CRE. As such, we determined whether OKA could induce promoter activity harbouring CRE elements. In cells transiently transfected with 4xCRE/tkCAT, which contains four consecutive CRE consensus sequences in front of (5′) a tkCAT construct, OKA induced a dose-dependent increase in CAT activity as compared to the untreated 4xCRE/tkCAT (Fig. 12A), an approximately 21-fold increase at 30 nM (mean, n = 3). OKA had only a marginal effect on the CAT activity of the thymidine kinase promoter/CAT construct (tkCAT, pBLCAT2) (Fig. 12A). KT-5720 at 2 μM, abolished greater than 90% (mean n = 3) of the CAT activity induced by OKA after 20 h of incubation.

Similarly, we determined whether OKA could transactivate an AP-1-dependent gene; in this regard, we chose the promoter region of c-*jun* (AP-1 site at −70) fused to a CAT reporter gene. After 20-h incubation period, OKA stimulated a greater than 50 fold increase (mean, n = 3) in CAT reporter activity which was abrogated to a very large extent when the AP-1 site was mutated (from wild-type 5′-GGTGACATCAT-3′ to 5′-GGATCCACCAT-3′) (Fig. 12B). Interestingly, phorbol ester (PMA) induced CAT activity by about sevenfold under the same experimental conditions (data not shown).

Effects of Pyrrolidinedithiocarbamate (PDTC), an Antioxidant, and KT-5720, an Inhibitor of PKA, on the OKA-Induced Synthesis of COX-2

Previous studies had indicated that OKA induced oxidative stress in certain cell types and

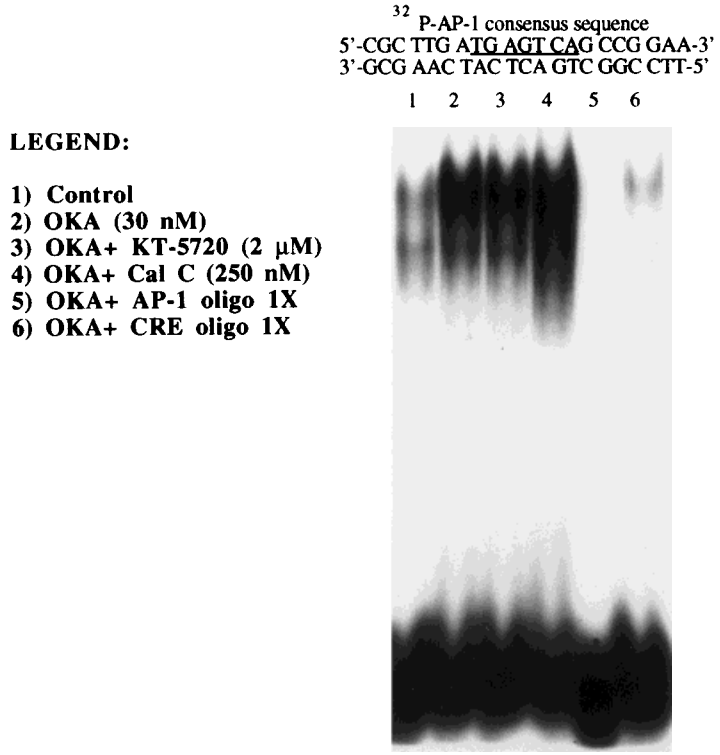

Fig. 8. OKA induction of AP-1 nuclear binding activity is not affected by protein kinase A (PKA) or protein kinase C (PKC)-mediated phosphorylation. Serum-starved, confluent quiescent chondrocytes were incubated without **(lane 1)** or with **(lane 2)** 30 nmol/L OKA in the presence of the specific PKA inhibitor KT-5720 (2 μM) **(lane 3)** or the specific PKC inhibitor calphostin C (CalC, 100 nM) **(lane 4)** for 20 h. Nuclei were isolated by hypotonic lysis of the chondrocytes and proteins extracted into high salt buffer. A total of 10 μg protein was incubated with ^{32}P-labeled AP-1 consensus sequence and subjected to electrophoresis on 6% polyacrylamide gels. **Lanes 5** and **6,** displacement of ^{32}P-labeled AP-1 consensus sequence nuclear binding activity with one-fold molar excess of radioinert AP-1 and COX-2 CRE.

that reactive oxygen species (ROS) could serve as common second-messenger-like molecules mediating the induction of many genes and the activation of transcription factors such as NF-κB [Schmidt et al., 1995]. With primary cultures of human chondrocytes, we explored the possibility that OKA-induced synthesis of COX-2 could be the result of oxygen radical formation by incubating OKA in the presence or absence of PDTC. The latter compound is a potent antioxidant and has been shown to block OKA-induced activation of NF-κB [Schmidt et al., 1995] in HeLa cells. As shown in Figure 13A, PDTC does not abrogate, to any significant extent, the OKA-induced synthesis of COX-2, nor does it have an effect on unstimulated levels of COX-2 synthesis.

In experiments reported above, we showed that OKA induced, in a delayed but pronounced manner, PKA. If the latter kinase plays any role in mediating the action of OKA in terms of COX-2 synthesis, then modifying its activity should affect the synthesis of the enzyme. As shown in Figure 13A, KT-5720, a potent inhibitor of PKA, blocks the synthesis and expression of COX-2 to a large extent. KT-5720 has a K_i value of approximately 50 nM for human PKA (Kemptide as substrate) [Gadbois et al., 1992] and can inhibit up to 90% of the PKA activity induced by OKA after 20 h in human chondrocyte cell extracts (cf. 745 ± 39 versus 65 ± 19 ng equivalents, mean = 3).

OKA Treatment of Chondrocytes Causes Little or No Increase in the Release of PGE_2

The production of PGE_2 is dependent on the concerted action of two principal enzymes phospholipase A2 (PLA_2), which liberates fatty acids (e.g., arachidonic acid) from the sn-2 position of phospholipids and cyclooxygenase (COX), which converts arachidonic acid to prostanoids, including PGE_2 [Piomelli, 1993; Dennis, 1997; Lin et al., 1992]. Prostanoid biosynthesis can be rapidly stimulated by Ca^{2+} mobilizing agents or more slowly, by proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [reviewed by Piomelli, 1993]. In a

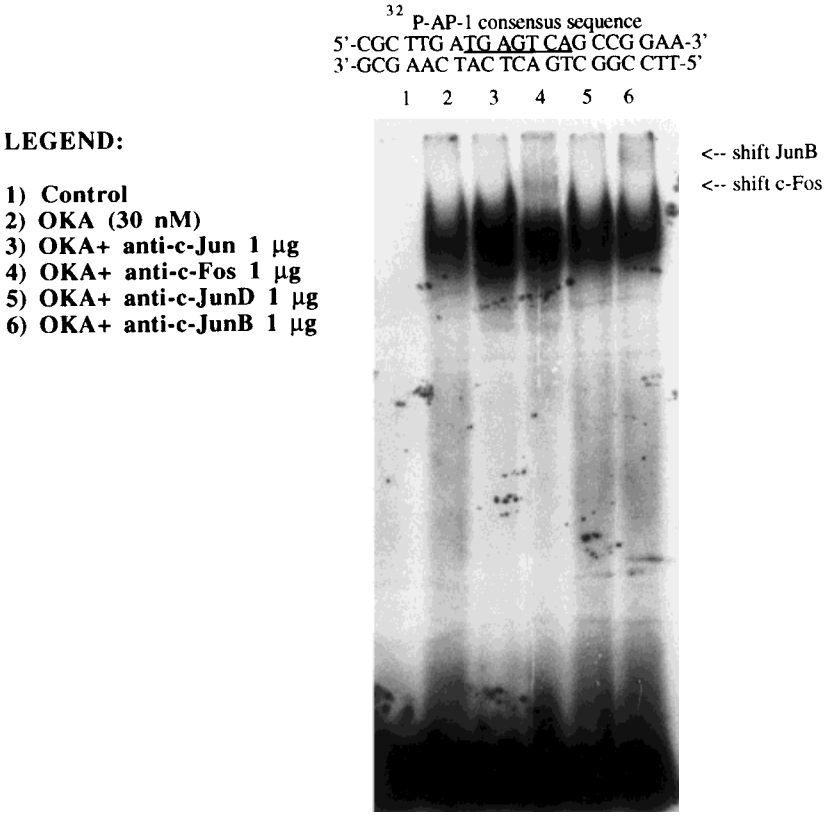

Fig. 9. Super gel-shift analysis of OKA induced AP-1 nuclear binding activity. Serum-starved, confluent quiescent chondrocytes were incubated without **(lane 1)** or with **(lane 2)** 30 nmol/L OKA for 20 h. Nuclei were isolated by hypotonic lysis of the chondrocytes and proteins extracted into high salt buffer. A total of 10 μg protein was incubated with ^{32}P-labeled AP-1 consensus sequence in the absence or presence of 1 μg each of anti-human antibodies to c-Jun, c-Fos, JunD, and JunB **(lanes 3–6)** as described under Materials and Methods. Binding complexes were resolved by nondenaturing PAGE through 6% gels in a Tris-borate buffer system at 4°C under low-voltage conditions.

culture system identical to one reported here, treatment of chondrocytes with IL-1β (100 pg/ml) or PMA (200 nM) caused an increase in COX-2 synthesis which coincided with a considerable increase in PGE_2 release reaching up to 410 ± 36.5 ng per million cells per 24 h with certain specimens (n = 4). Interestingly, staurosporine, a potent but nonspecific serine/threonine protein kinase inhibitor, had no effect on IL-1β-stimulated COX-2 synthesis, while calphostin C, a PKC inhibitor [Bruns et al., 1991], super-induced COX-2 synthesis. Treating chondrocytes with 30 nmol/L OKA caused only a very small increase in PGE_2 production (0.084 ± 0.009 ng/million cells/20 h) over controls (0.04 ± 0.03 ng/million cells/20 h, n = 3). These values represent 0.02% of what chondrocytes release in the presence of Il-1β or phorbol ester [Chan et al., 1995] despite a greater synthesis of COX-2 with OKA versus other inducing agents (Fig. 13B). Co-incubation of IL-1β (100 pg/ml) with OKA caused a slight decline in COX-2 synthesis (Fig. 13B).

DISCUSSION

While studying the regulation of COX-2 expression by inflammatory cytokines in primary cell cultures of human connective tissue origin, we examined the role of serine/threonine phosphatases using pharmacological inhibitors like OKA as a first approach. Initial observations revealed that OKA induced a massive (>50-fold with some specimens) upregulation of COX-2 gene transcription, expression and synthesis eclipsing all other known COX-2 inducers by far. With careful control of the concentration of OKA used, one could, with reasonable certainty, inhibit only PP-2A (K_i, 1 nM) and PP-1 (K_i, 10–15 nM) as has been shown by others [Cohen et al., 1990; MacKintosh and MacKintosh, 1994]. Thus primary (e.g., CREB-1) and secondary (PKC substrates) phosphatase target substrates would remain phosphorylated, perhaps even activated. If this were true, it would not be inconceivable that components of the signaling module involved in the COX-2 inductive process would be made conspicuous and quantifiable, given that the level of induction was so impressive.

The co-induction of members of the c-*jun* families of oncogenes was not surprising, as this is a feature of many cell lines treated with OKA. Despite some minor variations from one cell line to another, *jun*B and c-*jun* mRNA would

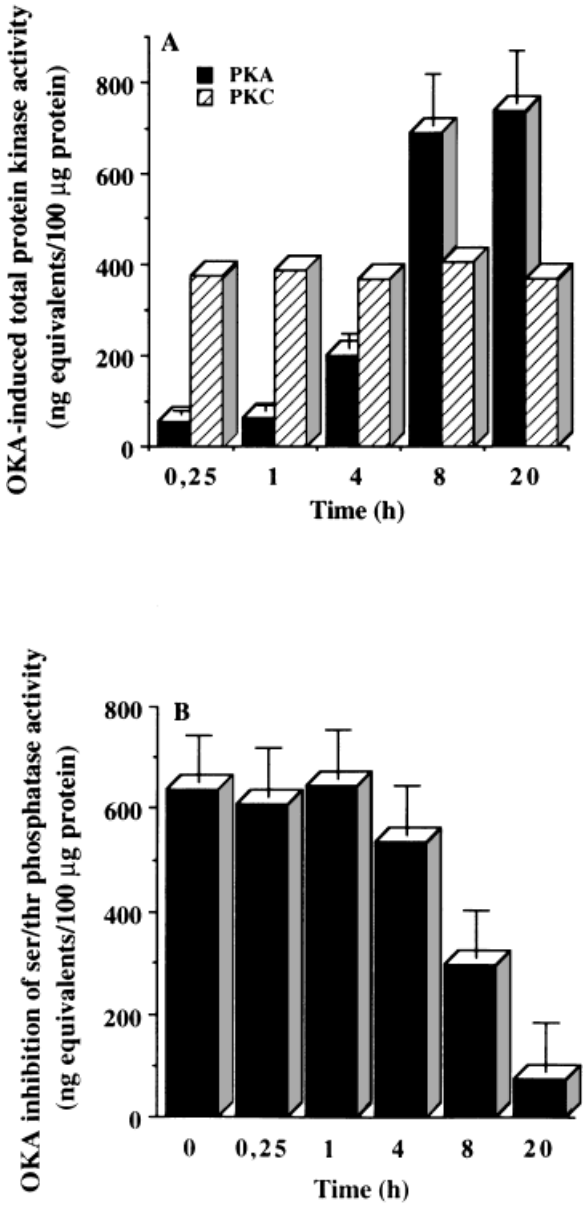

Fig. 10. Time course of OKA-induced effects on protein kinase A (PKA) and C (PKC) **(A)** and serine/threonine protein phosphatase activity **(B).** Normal chondrocytes were seeded at high density and confluence was reached in 1–3 days. The cells were then serum starved for 24 h and incubated with 30 nmol/L OKA (OKA) for the indicated times. Cellular proteins were extracted into RIPA buffer and analyzed for kinase and phosphatase activity as described under Materials and Methods. Standard curves (usually 0–100 ng) were generated with the purified catalytic subunit of PKA, a mixture of purified PKC isoforms, or purified protein phosphatase-1. Results are expressed as ng equivalents of total enzymatic activity per 100 μg of cellular protein; the change in optical density obtained with a fixed amount of cellular extract corresponding to an equivalent change in optical density for a given amount of purified enzyme. Values represent the means of three to four determinations in duplicate. Coefficient of intra- and interassay variation was always less than 11% and 19% at each time, respectively. $P = 0.0001$ for control (0) versus 20 h for PKA and PP-1.

seem to be the predominant forms induced by OKA and their patterns of expression were similar to COX-2 only appearing somewhat earlier. Indeed, in HT-1080 fibrosarcoma cells, JunB apparently mediates the induction of collagenase-1 by OKA [Westermarck et al., 1994]. However, JunB is not efficiently nor productively phosphorylated by SAPK/JNK, (whose activation is induced by OKA), nor does Jun B efficiently transactivate the collagenase promoter via a TRE element [Kallunki et al., 1996]. Jun B homodimers do not bind well to cognate regulatory elements [Ryseck and Bravo, 1991]; thus, if Jun B plays a role in COX-2 expression, it is likely the result of heterodimerization. Our supershift results implicated c-Fos, JunB, and less convincingly c-Jun, as components of the AP-1 nuclear binding species, but a more detailed approach will be required for more positive identification since complete shifts were never observed.

The up-regulation of c-*jun* expression is generally thought to occur through a TRE element (AP-1) in the proximal promoter region of the c-*jun* gene [Angel et al., 1988]. OKA would appear to activate the MEKK/SEK/SAPK/JNK cascade in human chondrocytes, and this would be consistent with what is known about the phosphorylation and the stimulation of the transactivation capacity of c-Jun. JNK activity was apprent after 1–2 h but was dramatically increased at 8 h. We detected an increase in AP-1 binding activity after a few hours, which preceded the increased expression of c-*jun*, consistent with an autoactivational process. Presumably, resident nuclear AP-1 complexes are phosphorylated/activated followed by c-*jun* gene transactivation [Angel et al., 1988]. Apparently, dimerization with ATF-2 is the favored dimer to bind to the TRE (AP-1) element of the c-*jun* gene as opposed to conventional AP-1 complexes [reviewed by Karin, 1995]. The c-*jun* TRE differs from consensus by the insertion of one base pair and resembles a CRE element which may be why members of the CREB family (like ATF-1/ATF-2) prefer this site. This could explain, in part, why we could displace nuclear binding to an ^{32}P-AP-1 consensus oligonucleotide with cold consensus CRE and COX-2 CRE oligonucleotides. Whether c-Jun/ATF complexes are prevalent in chondrocytes, or in connective tissue cells in general, remains to be determined.

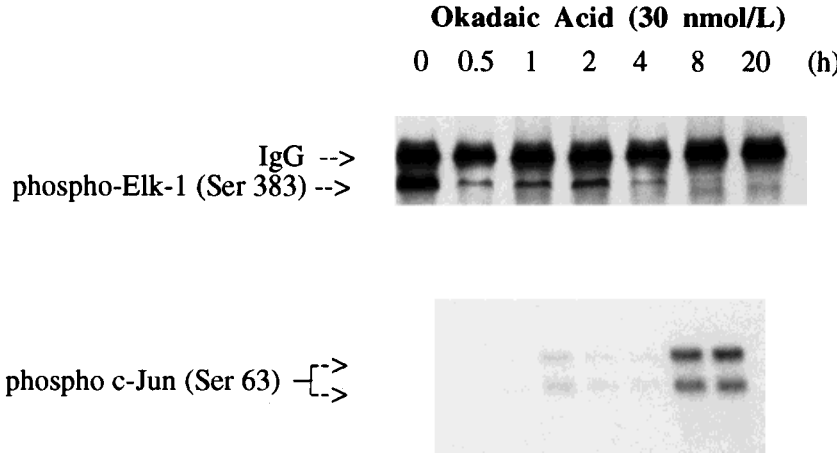

Fig. 11. Time course of OKA inhibition on mitogen-activated protein kinase (MAPK/ERK1,2) and stimulation of c-Jun NH terminal-stress activated protein kinase (SAPK/JNK). Serum-starved, confluent quiescent chondrocytes were incubated with 30 nmol/L OKA for the indicated times (0–20 h). Cellular proteins were extracted into RIPA buffer (plus phosphatase and protease inhibitors) and analyzed for MAPK/ERK1,2 (p42/44) and SAPK/JNK activity as described under Materials and Methods. Specific substrates were Elk-1 fusion protein, phosphorylated at ser 383 by ERKs and c-jun (1-89 a.a) GST fusion protein, phosphorylated by SAPK/JNK at ser 63 and ser 73. Detection of phosphorylated substrates was achieved using specific anti-phospho-substrate antibodies.

The induction of c-*fos* mRNA by OKA was perhaps quantitatively more pronounced than the c-*jun* family members but was alone among Fos proteins to be inducible, as we did not detect *fos*B, and Fra-1 (protein) was present constitutively. The promoter of c-*fos* contains a transcriptionally critical CRE and serum-response element (SRE) [Sheng et al., 1991; Treisman, 1986]. Our data are consistent with the notion that OKA up-regulated c-*fos* expression through the CRE for several reasons. First, SRE binds the transcription factor Elk-1, which is phosphorylated by ERK kinases. However, we demonstrate here that this cascade is inactivated by OKA treatment. By contrast, OKA upregulates PKA, the level of phosphorylated CREB-1/ATF-1 and CRE binding activity as judged by gel-shift analysis, leading us to surmise that c-*fos* was controlled, at least in part, from the CRE site.

We observed no stimulation of NF-κB activity with OKA in human chondrocytes, despite the fact such an effect has been demonstrated in other cell types [Schmidt et al., 1995]. In the latter scenarios, the induction was largely attributed to OKA-induced oxidative stress through the generation of reactive oxygen species. The effect could be prevented with the incubation of quenching antioxidants particularly in cells with transformed phenotypes. Our results, however, were substantiated by the observation that the same antioxidants used by the authors (PDTC) did not reverse OKA-induced COX-2 synthesis in our chondrocyte cultures, suggesting there is no association between NF-κB and OKA-induced COX-2 synthesis. The NF-κB site of human COX-2 promoter is implicated in the increase of enzymatic expression after IL-1β (and TNF-α) stimulation of human rheumatoid synovial fibroblasts [Roshak et al., 1996], the latter cells being of mesenchymal origin but phenotypically distinct from chondrocytes. PKC ζ was reported to play a mediating role in IL-1β-induced COX-2 expression in renal mesangial cells, possibly serving to activate the phosphorylation of IκB (and degradation with ubiquitin-proteosome) with a resultant migration of NF-κB to the nucleus [Rzymkiewicz et al., 1996]. The latter IL-1β induced signalling cascade was reported to be inhibited by calphostin C (a PKC inhibitor) but potentiated with staurosporine (a nonspecific but potent kinase inhibitor). In our hands, staurosporine had no effect on IL-1β-induced COX-2 synthesis in our chondrocyte culture system but calphostin C potentiated the action of the cytokine (Fig. 13B). Indeed, calphostin C, mimicking OKA, can potently upregulate COX-2 in chondrocytes, suggesting that the inhibition of PKC activity may inhibit phosphatase action, one that is dependent perhaps on phosphorylation by PKC for activity. It is possible that staurosporine acts the same way in renal mesangial cells as alluded to by the authors and our present data with OKA may be supportive in this regard.

Stimulation by OKA of PKA (activity/synthesis), phosphorylated CREB-1/ATF-1, CRE binding activity, and CRE transactivation of the thymidine kinase promoter, has not been previ-

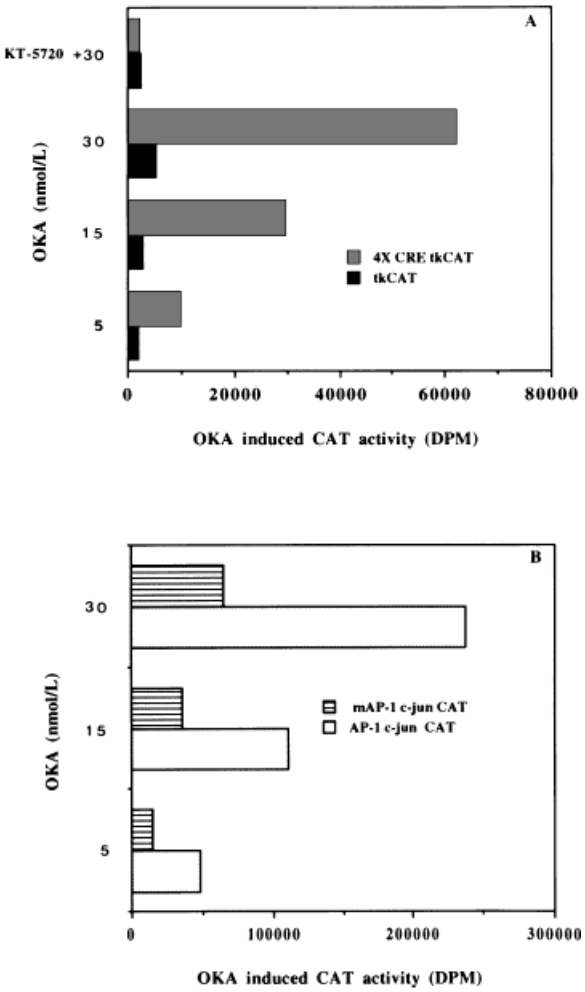

Fig. 12. Dose-response trans-activation by OKA of the thymidine kinase (tk) **(A)** and human c-jun **(B)** promoter via CRE and AP-1 regulatory sequences, respectively. Cells were co-transfected by the calcium phosphate precipitation method with 0.5 µg each of a plasmid expressing β-galactosidase (control of transfection efficiency) and the c-*jun*-CAT/mAP-1 c-*jun* CAT reporter plasmids (2 µg each) or the tkCAT/tkCAT-4X CRE plasmids (5 µg each). Cells were activated with increasing concentrations of OKA (0–30 nmol/L) as indicated for 20 h. Cells were lysed in buffer containing Triton X-100 and the lysates assayed for β-galactosidase (colorimetry) and CAT activity using the liquid scintillation counting method. The c-*jun*-CAT reporter plasmid was constructed by inserting a −132/+170-bp fragment from the promoter region of the human c-*jun* gene into the pBLCAT3 vector upstream from the CAT gene. A second reporter plasmid, identical to the previous one, except that the AP-1 site (5′-GGTGACATCAT-3′) was mutated to 5′-GGATCCACCAT-3′, was used to determine the role of the AP-1 site in the induction of c-*jun* promoter activity by OKA. KT-5720 (2 µmol/L), a specific inhibitor of protein kinase A (PKA) was added to determine the role of PKA in the mediation of promoter activation by OKA at the CRE site. Values represent the means of three determinations in duplicate. Intra- and interassay coefficients of variation reached a maximum of 9% and 16%, respectively. Probability values (*P*): control (0 h) versus 30 nmol/L OKA with background (mAP-1 c-*jun* CAT or tkCAT) subtraction was ≥0.0001.

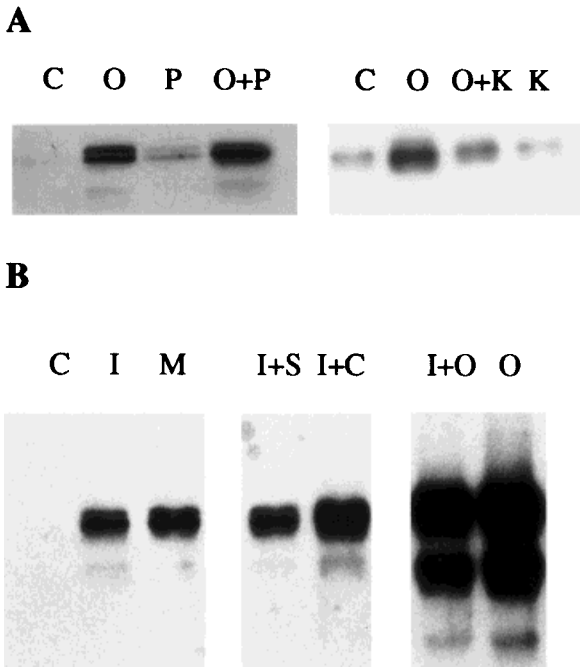

Fig. 13. OKA induction of COX-2 enzyme synthesis is inhibited by KT-5720, but not by the anti-oxidant, PDTC. **A:** Quiescent cultured chondrocytes were incubated with 30 nmol/L OKA for 20 h in the presence or absence of 100 µmol/L pyrrolidine dithiocarbamate (P) or of 2 µmol/L PKA inhibitor, KT-5720 (K). Cellular proteins were extracted into RIPA buffer and analyzed by Western blotting, using a specific anti-COX-2 antiserum as described under Materials and Methods. **B:** Quiescent cultured chondrocytes were stimulated without (control, C) or with 100 pg/mL (5.7 pmol/L) of rhIL-1β (I) in the presence or absence of the kinase inhibitors staurosporine (S, 10 nmol/L) and calphostin C (C, 100 nmol/L) or OKA (O, 30 nmol/L) for 20 h. Phorbol ester (M) was incubated for 20 h alone at 200 nmol/L. Note the massive induction of COX-2 enzyme synthesis by OKA with this representative cell line.

ously observed and may be a human chondrocyte specific response. Further, the latter occurred in the absence of any measurable increase in cAMP. Although seemingly out of the ordinary, it has been shown that IL-1β, example, can activate PKA in AtT-20 cells without an increase in cAMP [Gwosdow et al., 1993]. Thus pursuant to further studies, we surmise that the increase in total PKA activity represented increased PKA synthesis since PKA activation is not dependent on phosphorylation. Additional information on the relationship between PKA and COX-2 expression came from experiments with KT-5720, which avidly (K_i, 50 nmol/L) inactivates PKA [Gadbois et al., 1993], and which inhibited to similar degrees the expression of COX-2 and CRE binding activity. Furthermore, AP-1-binding activity was not af-

fected by pretreatment of cells with KT-5720, as expected, since PKA does not phosphorylate AP-1 [Hunter and Karin, 1992]. In well studied systems, the extent of Ser-133 phosphorylation of CREB , corelates directly with the rate of transcription of cAMP-responsive genes [Hagiwara et al., 1993]. Such phosphorylation is believed to favour the association of CREB-binding protein (CBP), a coactivator that can interact with TFIIB [Kowk et al., 1994]. When we coincubated an antibody to CBP with OKA-induced nuclear extracts, ^{32}P-CRE binding was not shifted but eliminated. In the absence of a trivial explanation, these results could be interpreted to mean that CREB binding to a cognate CRE requires association to CBP.

Phospho (Ser-133) CREB serves as an important substrate for PP-1 and PP-2A, although the latter phosphatase may be more active [Hagiwara, 1992]. In our culture system, the time course of OKA inhibition of PP-1/PP-2A preceded the appearance of phosphoCREB and our dose-response studies tend to implicate PP-1.

It would not be totally unreasonable to implicate AP-1 in the transactivation of the COX-2 gene, even in the absence of canonical or recognizable AP-1 elements. First, besides CRE binding, AP-1 was the only other significant transactivating factor detected in our gel-shift assays that could potentially recognize regulatory elements (e.g., CRE) positively identified in the promoter region of the COX-2 gene. ^{32}P-AP-1 oligo was displaced by a radioinert COX-2 CRE site further suggesting the possibility of AP-1 transactivation through a CRE site. In v-*src*-transformed 3T3 cells, Xie and Herschmann [1995] proposed that the signal pathways leading to *src*-induced COX-2 transcription involves both Ras/MEKK-1/JNK and Ras/Raf-1/ERK cascades. Further, these investigators proposed that AP-1 (c-Jun) transactivates through the CRE element. In our terminally differentiated chondrocytes, however, the Ras/Raf-1/ERK pathway appears not to be involved. The suppression of the latter signalling module coincides temporally with the appearance of increased PKA activity, consistent with previous studies showing PKA inhibition of Raf in NIH 3T3 cells [Cook and McCormick, 1993].

Using a different experimental approach from this present study involving the transfection of various COX-2 promoter constructs into monocytic [Inoue et al., 1994] and vascular endothelial cells [Inoue et al., 1995], the importance of NF-IL-6 and CRE consensus sequences in mediating the induction of COX-2 expression by LPS and phorbol ester was shown. Though our results are in general agreement, we could find no NF-IL-6-COX-2 site-binding activity (i.e., C/EBP) in OKA-stimulated nuclear chondrocyte extracts and NF-IL-6-COX-2 oligos did not displace ^{32}P-CRE from nuclear binding proteins. Our transfection studies indicate that OKA can transactivate a c-*jun*-CAT promoter construct through an AP-1 site, which would be consistent with our observed increases in c-*jun* expression and AP-1 DNA-binding activity.

The massive OKA-induced increase in COX-2 gene transcription, mRNA expression, and COX-2 enzyme synthesis in the absence of a concomitant release of PGE_2 suggests that $cPLA_2$ expression and activity may also be influenced by the cellular level of serine/threonine phosphatase activity. The role of $cPLA_2$ in human chondrocyte release of PGE_2 after treatment with IL-1β is unresolved as there was no correlation reported between PGE_2 production and $cPLA_2$ activity [Knott et al., 1994]. Activation of $cPLA_2$ requires serine phosphorylation by ERK1/2 and Ca^{2+} [Piomelli, 1993; Dennis, 1997] for maximum induction and, as such, one would expect sustained phosphorylation of $cPLA_2$ in the presence of OKA and thus increased activity. This notion must be tempered by the fact that OKA inhibits ERK activity, suggesting that, over the long term, OKA may inhibit $cPLA_2$ activity and reduce arachidonic acid production, the substrate for COX-2. Interestingly, long-term incubations (>30–36 h) of human chondrocytes with OKA results in a process akin to apoptosis, which can be reversed with the addition of high nanomolar concentrations of PGE_2 (unpublished observations). These observations were unique to OKA probably because other agents that induce COX-2 also stimulate large quantities of PGE_2. Thus previous reports linking COX-2 expression/synthesis with apoptosis in cells with transformed phenotypes [Tsujii and DuBois, 1995] may also apply to primary cells that are stable and terminally differentiated.

We may conclude that, in human chondrocytes, the COX-2 gene may be controlled in part by serine/threonine protein phosphatases like 1 and 2A; OKA has never been shown to inhibit

other known phosphatases and Calyculin A gave essentially the same results [Cohen et al., 1990; MacKintosh and MacKintosh, 1994]. The inhibition of PP-1/PP-2A results in a shutdown of MEKK1/MEK1/ERK cascade probably as a result of an increase in PKA activity, which is known to inhibit Raf-1, a MEKK1 kinase. By contrast, there is a concomitant activation of the other proline-directed MAP kinase pathway, namely, MEKK1/JNKK/SAPK/JNK, in addition to increased levels of PKA. There is circumstantial evidence in this study to implicate a Fos kinase as well, given the prominence of c-Fos in the AP-1 complexes. The accumulated AP-1 factors probably consisted, in part, of c-Fos/c-Jun, c-Jun/JunB and c-Fos/JunB dimers as judged by supershift assays but we require further experiments to complete the identification process. The AP-1 and CREB/ATF family of transcription factors may be substrates for PP-1/PP-2A in human chondrocytes. Additional studies are under way to identify the regulatory elements in the promoter region of the COX-2 gene that mediate increases in the rate of COX-2 gene transcription following phosphatase inhibition. Okadaic acid, and like substances, can indeed be a useful chemical tools in certain situations and it has allowed us to better understand how COX-2, the target of virtually all nonsteroidal anti-inflammatory drugs (NSAIDs), is regulated in a primary nontransformed cell type.

ACKNOWLEDGMENTS

This research was supported by grants (J.D.B.) from the Canadian Arthritis Society and the Medical Research Council of Canada.

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