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Calphostin C Induces AP1 Synthesis and AP1-Dependent c-*jun* Transactivation in Normal Human Chondrocytes Independent of Protein Kinase C-α Inhibition: Possible Role for c-*jun* N-Terminal Kinase

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Abstract Activator protein-1 (AP1) regulates the promoter activity of a large number of genes associated with developmental, proliferative, inflammatory, and homeostatic processes in human connective tissue cells. Some of these genes (e.g., cyclooxygenase-2) are regulated by the protein kinase C (PKC) inhibitor, calphostin C (CalC). We examined whether CalC could indeed induce AP1 and AP1 gene transactivation (c-*jun*) in human chondrocytes. Exploratory studies confirmed the anti-PKC effects of CalC, as equal molar concentrations of CalC blocked the PMA-induced translocation of PKC-α from the cytosolic to the membrane fraction. CalC induction of AP1, as judged by gel-shift analysis, using a consensus AP1 oligonucleotide, was biphasic with an initial increase (maximum 4 h), followed by a decline, reaching its nadir after 16 h, and finally a major upregulation phase at 24 h. Maximum induction of AP-1 was reached at a concentration of 250 nmol/L of CalC. CalC did not block PMA-induced AP1 synthesis. Gel-shift analysis in the presence of specific antibodies to c-Jun, JunB, JunD, c-Fos, and CREB/ATF showed that the AP1 complexes were probably c-Jun/c-Jun, c-Fos/c-Jun, c-Fos/JunB, or c-Jun/JunB dimers. Northern blot analysis confirmed that c-*jun*, *jun*B, and c-*Fos* were the principal proto-oncogenes induced by CalC. To confirm that c-*jun* induction occurs at the transcriptional level and to examine the role of the AP1 site present in the c-*jun* promoter in the induction of c-*jun* by CalC, we performed transient transfections of c-*jun* promoter-CAT constructs harboring either wild-type (WT) AP1 regulatory element sites or mutant AP1 sites. CalC (250 nmol/L) induced a marked increase in CAT activity (i.e., promoter activation) with WT AP1 c-*jun* promoter-CAT plasmids, but the response was completely abrogated when using constructs where the AP1 site was mutated. PMA produced similar results, but the induction of the WT AP1 c-*jun* promoter-CAT plasmid was smaller. CalC (250 nmol/L) inhibited MAPK (p42/44) activity while stimulating c-Jun N-terminal kinase activity in a time-frame coincident with the activation of AP1. We conclude that CalC induces signaling pathways that activate AP1 and transactivate genes harboring AP1 enhancer sites independent of PKC-α. J. Cell. Biochem. 76:290–302, 1999.

Key words: chondrocytes; Calphostin C; c-Jun N terminal kinase; AP1; protein kinase C

Activator protein-1 (AP1) is a DNA sequence-specific transcriptional activator and an important mediator of cellular responses to growth factors, serum, and tumor-promoting phorbol esters [Angel et al., 1987; Curran and Franza, 1988]. AP1 is a dimeric protein consisting of c-Jun (c-Jun, JunB, JunD) and c-Fos (Fra-1,2, FosB, c-Fos) family members that enhances gene transcription in promoters harboring the so-called phorbol ester response element (TRE), having the consensus sequence TGA(C/G)TCA [Curran and Franza, 1988; Angel et al., 1988; Nakabeppu et al., 1988]. Whereas c-Jun proteins can form stable homodimers, c-Fos proteins cannot and must complex with c-Jun fam-

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ily members. Furthermore, c-Jun proteins can heterodimerize with CREB/ATF family members as well, and the latter heterodimers can transactivate target genes at TRE sites [Benbrook and Jones, 1994; Gupta et al., 1995].

After induction by phorbol ester or ultraviolet (UV) light, AP1 binds rapidly (30 min to 2 h) to the TRE and increases promoter activity in target genes (e.g., c-*jun*, collagenase) without any increase in the synthesis of c-Jun or c-Fos [Angel et al., 1987]. This is the result of post-translational modifications involving the specific phosphorylation of amino acid residues (e.g., N-terminal ser-63 and ser-73) of c-Jun and c-Fos (C-terminal thr 232) that endow the proteins with increased transcriptional activity [Curran and Franza, 1988]. The c-*jun* promoter has a rather uncomplicated structure with most of its inducers operating through the c-*jun* TRE. Activation of AP1 leads to auto up-regulation of the c-*jun* gene through the TRE site and amplification of the original signal [Angel et al., 1988].

AP1 phosphorylation may be accomplished by a group of proline-directed mitogen-activated protein kinases (MAP) [Karin, 1995; Hunter and Karin, 1992] called Jun N-terminal, stress-activated kinase (JNK/SAPK). c-Fos, in fact, is not a good substrate for JNK; rather, it is phosphorylated specifically and productively by the Fos-regulating kinase (FRK) [Deng and Karin, 1994]. Depending on the cell type, the activity of JNK may be controlled by Ca^{2+} and protein kinase C (PKC)-dependent pathways [Su et al., 1994]. Protein kinase C (PKC) represents a family of isoenzymes with at least 11 members, categorized as conventional (4) and novel (8) isoforms, and are single polypeptides composed of an N-terminal regulatory region and a C-terminal catalytic domain [Dekker et al., 1995]. Maximizing the activity of conventional isoforms of PKC is dependent on the presence of Ca^{2+}, phospholipids (principally phosphatidylserine [PS]) and diacylglycerol (DAG), which in turn are generated in the cell by a diversity of transduction mechanisms promoting lipid hydrolysis [Nishizuka, 1992; Kiley and Jaken, 1994; Newton, 1995]. Phorbol esters are potent inducers of PKC by virtue of their affinity for the DAG site in the regulatory domain of the enzyme [Newton, 1995]; these observations have both directly and indirectly linked PKC activation to AP1 phosphorylation and promoter transactivation [Su et al., 1994].

Although the regulatory mechanisms are quite complex, involving a number of steps, including multiple auto- and transphosphorylation, a hallmark of conventional PKC activation is the translocation of the mature form from the cytoplasmic compartment to the membrane [Newton, 1995]. Membrane translocation is mediated by DAG (and phorbol esters) and PS binding to the cognate domains in the enzyme [Kiley and Jaken, 1994; Newton, 1995]. PKC activation and translocation can be blocked by a group of highly selective PKC inhibitors such as Calphostin C (CalC). Calphostin C is a polycyclic hydrocarbon (perylenequinones) isolated from *Cladosporium cladosporioides* that binds specifically and with high affinity (IC_{50} = 50 nmol/L) to the DAG site in the regulatory (unique) domain of PKC [Kobayashi et al., 1989; Bruns et al., 1991]. The inhibitor has been used extensively and has proved a useful probe in the study of PKC-dependent signaling pathways and gene expression [Das and White, 1997; Xu and Clark, 1997]. Furthermore, it has been cited for its potential as a useful drug in cancer treatment and various forms of arthritis because it is pro-apoptotic in many cell types [Lee et al., 1995; Yang et al., 1995; Kobayashi et al., 1989]. A biochemical rationale for the action of CalC in this regard remains to be clarified although proto-oncogene expression (e.g., c-*jun*) has been implicated [Gamou and Shimizu, 1994; Freemerman et al., 1996].

While studying the role of PKC-α (major conventional isoform in chondrocytes) in the control of cyclooxygenase-2 (COX-2) gene expression in human chondrocytes [Miller et al., 1998; and unpublished observations], we observed that CalC mimicked the stimulatory effects of the phorbol ester, phorbol-12-myristate-13-acetate (PMA). When co-incubated, CalC and PMA produced additive effects in terms of COX-2 expression in human phenotypically stable chondrocytes.

Because AP1 is known to mediate gene induction of COX-2 [Xie and Herschman, 1995; Miller et al., 1998] in many cell types, we sought to determine whether CalC could indeed up-regulate AP1 expression and synthesis, TRE binding, and AP1 transactivation of c-*jun* gene through the major TRE site, as a rationale to explain some of the biochemical effects of CalC.

MATERIALS AND METHODS

Chemicals

Diethylpyrocarbonate (DEPC), polyvinylpyrrolidone (PVP), Ficoll, salmon testes DNA, ethylenediamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), sodium acetate, ethidium bromide (EtBr), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Calphostin-C and PMA were supplied by Calbiochem (San Diego, CA). Bio-Rad (Mississauga, ON) supplied acrylamide, bisacrylamide, ammonium persulfate, tetramethylethylenediamine (TEMED), bromphenol blue, Coommassie 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 of penicillin base, 10,000 µg/ml of streptomycin base, and 50 µg/ml of amphotericin-B); agarose and phenol were obtained from Gibco-BRL (Gaithersburg, MD). Fisher-Scientific (Montreal, Quebec, Canada) 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 was 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 underwent further processing. 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 β). 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, 20 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., 1994]. The following probes, labeled with digoxigenin (DIG)-dUTP by random priming, were used for hybridization. Human *jun*B and D were 1.6 and 1.0 *Eco*RI and *Acc*I/*Eco*RI cDNA fragments, respectively, from pBluescript SK− and were kindly provided by Dr. D. Skup (Louis-Charles Simard Research Center, Montreal, Quebec, Canada). The mouse c-*jun* 1.8-kb cDNA probe, provided by Dr. D. Edwards (University of Calgary, Alberta, Canada), 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, University of Caen, France) and was excised for labeling using *Pst*I and *Bcl*I. The mouse *fos*-B 1.6 kb was kindly provided by Dr. D. Skup and the human *fra-1* 40-mer DNA probe was obtained from Geneka Biotech Inc. (Montreal, Quebec, Canada) All nonhuman probes were previously shown to hybridize specifically with human mRNA [Miller et al., 1998]. 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). Calphostin C modulates 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 increases to 125–140% of the control values after 24 h (data not shown).

Preparation of Cell Extracts and Western Blotting

Isolation of cytosolic and particulate fractions of PKC was conducted according to previously described methods [Rzymliewicz et al., 1996]. 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% Nonidet P-40 (NP-40), 1 mM sodium orthovanadate, and 1 mM NaF from control and treated cells was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through 10% 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 room temperature (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 RT and then washed extensively for 30–40 min with TTBS, with 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. PKC isoenzyme antibodies are products of Santa Cruz Biotechnology (Santa Cruz, Biotech Inc., CA) and were used at dilutions suggested by the manufacturer.

Gel-Retardation Experiments

Control and treated chondrocytes in 4-well cluster plates (3–5 $\times$ 10^6 cells/well) were carefully scraped into 1.5 ml of ice-cold phosphate-buffered saline (PBS) and pelleted by brief centrifugation as previously described [Ney et al., 1990]. The cellular pellet was gently resuspended in 200–400 µl of 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% NP-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 use.

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}P]ATP using T4 polynucleotide kinase (Pharmacia, Montreal, Quebec, Canada). The sense sequences of the oligos used were as follows: AP-1 consensus; 5′-CGC TTG ATG AGT CAG CCG GAA-3′: AP-1 c-*jun*; 5′-CGC TTG ATG AGAT CAG CCG GAA-3′: NF-κB consensus; 5′-AGT TGA GGG GAC TTT CCC AGG C-3′: CRE; 5′-AGA GAT TGC C TG ACG 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′: c/EBP; 5′-CAC CGG GCT TAC GCA ATT TTT TTA A-3′: OCT-1; 5′-TGT CGA ATG CAA ATC ACT AGA 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 of 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. Binding complexes were resolved by nondenaturing polyacrylamide gel electrophoresis through 6% gels in a Tris-borate buffer system, after which the gels were fixed, dried, and prepared for autoradiography. For gel-shift analysis in the presence of anti-transcription factor antibodies, nuclear extracts were preincubated with 2 µg of antibodies (Santa Cruz Biotech) against human c-Jun, JunB, JunD, c-Fos, Fra-1,2, and CREB/ATF-1 for 20 min at RT before the addition of the radiolabeled oligonucleotide. All antibodies were raised against the C-terminus of the respective human protein except Fra-1, for which the epitope maps to the N-terminus of rat protein, although it has been shown to recognize human Fra-1.

Analysis of Protein Kinase Activity

Chondrocytes were incubated with CalC (250 nM) for 0–48 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. Generally 20–40 µg of protein was used per assay (linear range 0–100 µg).

The mitogen-activated protein kinase (MAPK, p 44/42) 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 MAPKs (Tyr204) were used to selectively immunoprecipitate active MAPKs (p42/44, ERK-1, and ERK-2) from chondrocyte lysates; 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 selectively to pull-down SAPK/JNK from chondrocyte 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].

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 CalC (250 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 ΔAP1 *jun*-CAT, where the wild-type (WT) AP1 consensus sequence is mutated (gift from Dr. M. Karin). CAT assays were measured by a specific CAT ELISA (Boehringer Mannheim, Québec, Canada). CAT values, as reported, were normalized to the level of β-galactosidase activity in each sample.

Statistical Analysis

Where appropriate results are expressed as mean ±SD of two to five separate experiments. Statistical significance was assessed using Student's *t*-test, and significant differences were confirmed only when the probability was ≤5%.

RESULTS

Calphostin C (CalC) Inhibits PMA-Induced PKC-α Translocation in Human Chondrocytes

We previously reported that CalC inhibits PMA-induced PKC activity in human chondrocytes in culture [Di Battista et al., 1996]. With the use of specific antibodies (conventional PKC-α, βI, βII, γ), we determined that PKC-α is the major conventional identifiable isoform in chondrocytes on a quantitative basis, although there were trace levels of PKC-βI (data not shown). In order to test the response of chondrocytic PKC-α to phorbol ester treatment, primary human chondrocytes were incubated with 300 nmol/L of PMA for 0–60 min. PMA (300 nmol/L) induced a time-dependent depletion of the PKC-α protein from the cytosolic fraction that began after 2–3 min and was nearly complete after 60 min. There was a concomitant reciprocal accumulation of PKC-α in isolated membrane fractions (Fig. 1A). When CalC (300 nmol/L) was preincubated with chondrocytes in culture for 1 h before the addition of PMA, PKC-α translocation was largely prevented (Fig. 1B).

Calphostin C (CalC) Induces AP1 Activity in Nuclear Extracts

CalC induction of AP1, as judged by gel-shift analysis using a consensus AP1 oligonucleotide, was biphasic with an initial cycloheximide insensitive increase (maximum 4 h), followed by a decline reaching its nadir after 16 h, and finally a major upregulation phase at 24 h (Fig. 2A, top and bottom). Maximum induction of AP1 was reached at a CalC concentration of 250 nmol/L, as judged by laser scanning densitometry of multiple gel shifts (Fig. 2B). PMA (300 nmol/L) increased AP1 binding activity, but this latter response was not inhibited by preincubation for 1 h with CalC (250 nmol/L) (Fig. 2C). Radioinert AP1/TRE consensus oligonucleotide (Fig. 2B, lane 7) and c-*jun* AP1/TRE oligonucleotide (Fig. 2C, lane 5) displaced the shifted bands

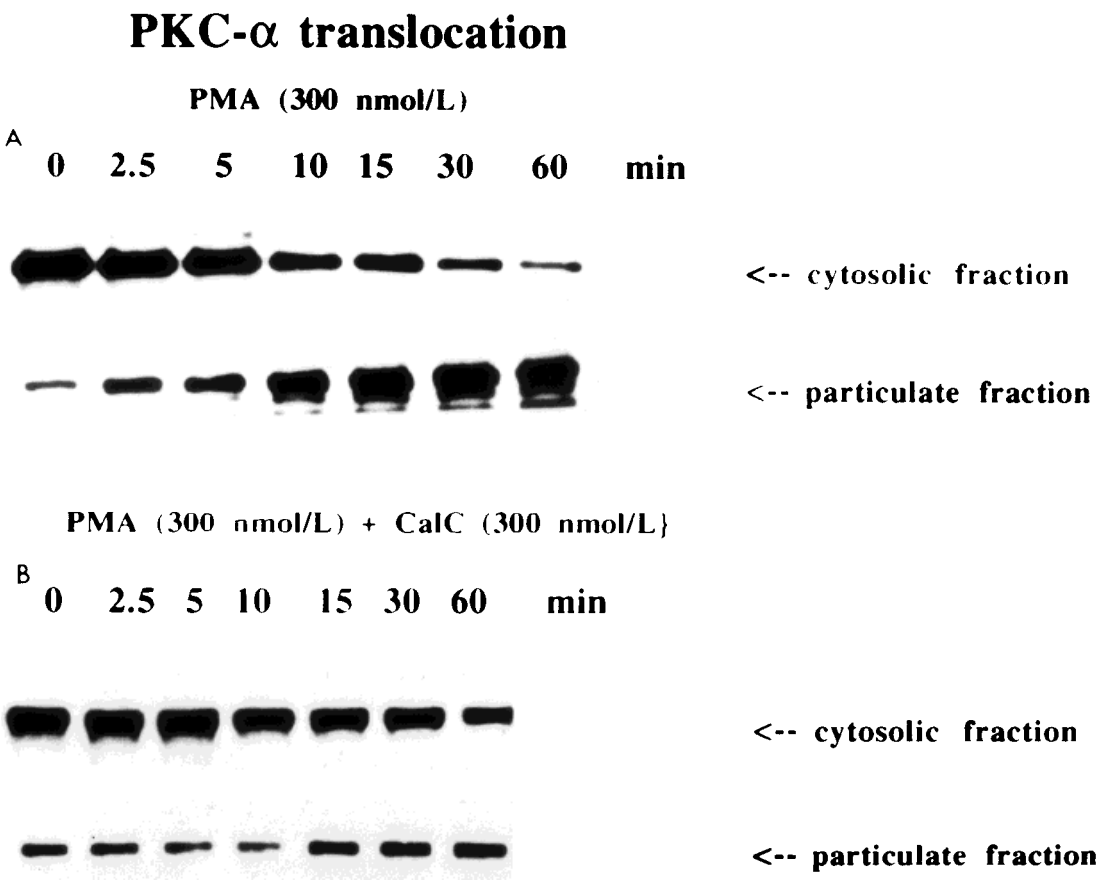

Fig. 1. Calphostin C (CalC) inhibits phorbol myristate acetate (PMA)-induced PKC-α translocation in human chondrocytes. Normal human chondrocytes were seeded at high density (1.2×10^6 cells per well), and confluence (stationary phase) was reached in 1–3 days. **A:** Cells were then serum starved for 24 h and then incubated with 300 nmol/L of PMA for 0, 2.5, 5, 10, 15, 30, and 60 min. **B:** 300 nmol/L of CalC was preincubated with the cells 1 h before the addition of PMA. Cellular proteins from the cytosolic and particulate fractions were extracted as described under Materials Methods and analyzed by Western blotting, using a specific anti-human PKC-α antiserum (1:1,000 dilution).

completely. Gel-shift analysis in the presence of specific antibodies to c-Jun, JunB, JunD, c-Fos, and CREB/ATF-1 demonstrated that the AP1 complexes were probably c-Jun/c-Jun, c-Fos/c-Jun, c-Fos/JunB, or c-Jun/JunB dimers as the AP-1 shift patterns were displaced by these respective antibodies, and not by JunD or CREB/ATF-1 antibodies (Fig. 2D). Co-incubation with anti Fra-1 or Fra-2 antibodies produced little or no effect in shift patterns (data not shown). There were no "supershift" patterns to slower moving complexes as such suggesting that these antibodies compete or titrate their cognate proteins and reduce homo/heterodimer formation and binding to the AP1 oligomer. Treatment of chondrocytes with CalC did not significantly increase binding of nuclear extracts to NF-κB, c/EBP, SP1, OCT-1, or AP2 consensus oligonucleotides but caused biphasic shift patterns with ^{32}P-labeled CRE oligos very similar to that of the AP-1 illustrated in Figure 2A (data not shown).

Calphostin C (CalC) Stimulates c-*jun*, *jun*B, and c-*Fos* mRNA Expression

Although AP1 complexes are often composed of the gene products of c-*jun* and c-*fos*, other members of the latter proto-oncogene families may be present as well. We wished to confirm and extend our studies on the identity of the AP1 complexes by mRNA analysis of CalC-treated chondrocytes. Calphostin C upregulated the expression of c-*jun* and c-*fos* proto-oncogene mRNA expression (Fig. 3) in a gradual time-dependent fashion, with the zenith reached at 8–16 h and declining thereafter (Fig. 3). *Jun*B basal expression was more elevated than the latter two oncogenes but also increased

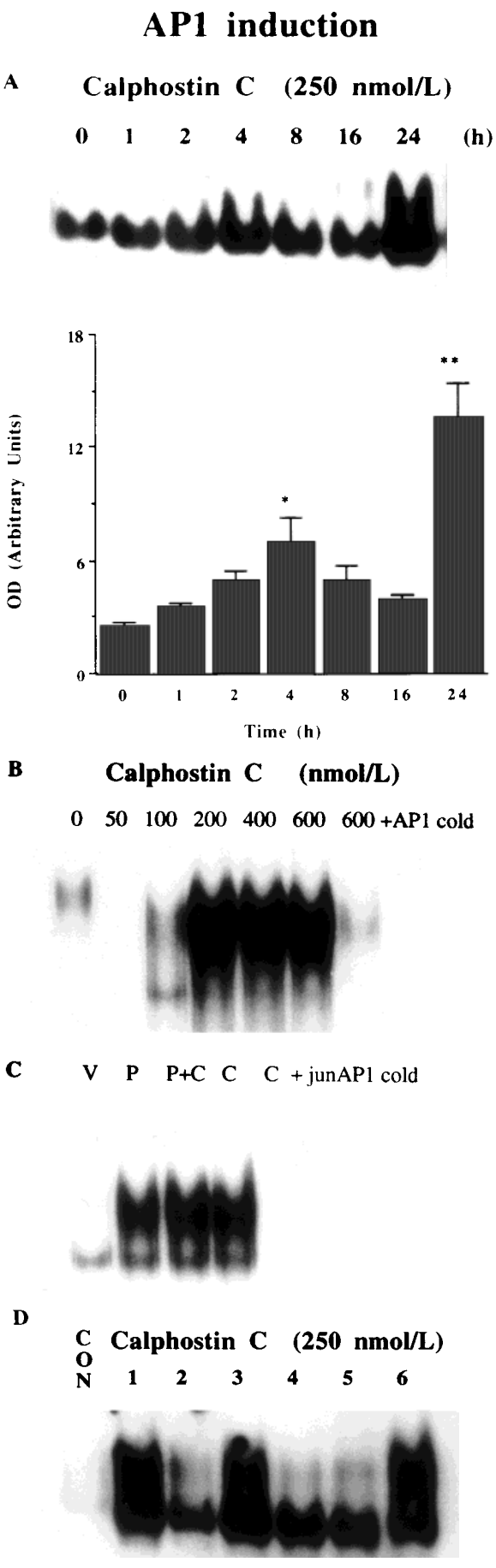

Fig. 2. Time course and dose-response of CalC induction of AP-1 consensus sequence nuclear binding activity. Normal chondrocytes were seeded at high density, and confluence was reached in 1–3 days. Cells were then serum starved for 24 h and then incubated with 250 nmol/L of CalC for 0, 1, 2, 4, 8, 16, and 24 h. **A:** Top, gel-shift pattern. Bottom, densitometric scan of four experiments. Dose-response experiments (**B**) were conducted over a 24-h period with 0, 50, 100, 200, 400, and 600 nmol/L. **C:** Cells were incubated with vehicle, phorbol myristate acetate (PMA) (P) 300 nmol/L, PMA 300 nmol/L + CalC (C) 250 nmol/L, or CalC 250 nmol/L for 24 h. **D:** Cells were incubated in the presence or absence of 250 nmol/L of CalC for 24 h. Nuclei were then 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 of nuclear protein was incubated with ^{32}P-labeled AP1 consensus sequence. **B:** From left, **lane 7,** 1-fold molar excess of radioinert homologous AP1 (TRE) consensus oligonucleotide was added to the reaction mixture. **C:** From left, **lane 5,** 1-fold molar excess of radioinert c-*jun* AP1 (TRE) oligonucleotide was added. **D:** From left, **lanes 3, 4, 5, 6, 7,** nuclear extracts were preincubated for 20 min at room temperature (RT) with 2 μg of antibodies against human c-Jun, JunD, c-Fos, JunB, and CREB/ATF-1, respectively, before the addition of radiolabeled probe as described under Materials and Methods. Mobility shifts were verified on 6% polyacrylamide gels run at 4°C, followed by gel drying and autoradiography. *4 h vs control, $P < 0.03$, n = 4: (**) 24 h vs control, $P < 0.0075$, n = 4.

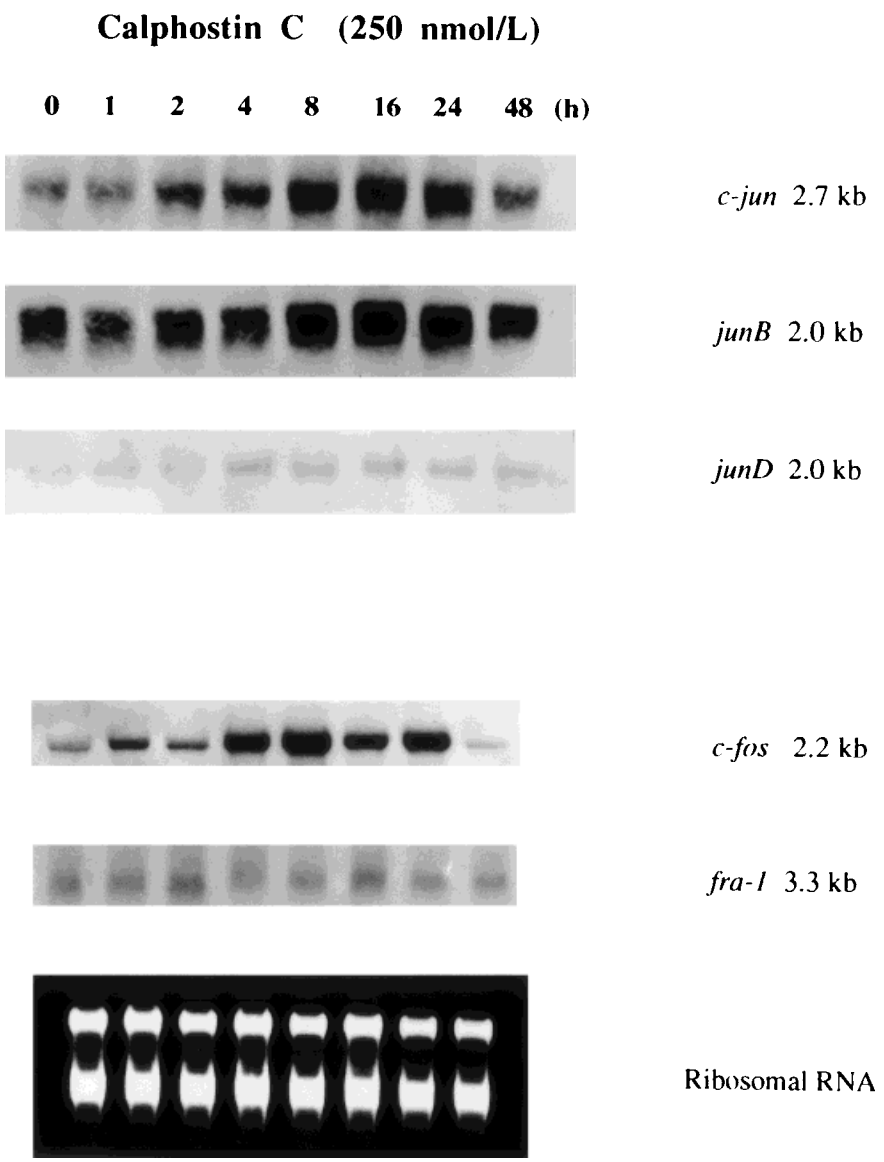

Fig. 3. Time course of CalC induction of members of the c-*jun* and c-*fos* families of proto-oncogenes. Serum-starved confluent quiescent chondrocytes were incubated with 250 nmol/L of CalC for 0, 1, 2, 4, 8, 16, 24, and 48 h as indicated. RNA was extracted using the Trizol reagent; 20 µg was analyzed by Northern blotting, using specific probes for c-*jun, junB, junD, fra-1,* and c-*fos.* The ethidium bromide staining of ribosomal RNA is representative of multiple blots used to assess mRNA levels for the proto-oncogenes.

gradually, with a peak observed at 16–24 h, followed by a decline (Fig. 3). There was no similar upregulation of *Jun*D or *fra*-1 mRNA (Fig. 3), and cycloheximide treatment did not inhibit c-*jun* mRNA expression but, in fact, induced a small superinduction (data not shown).

Induction of c-*jun* Promoter Constructs by Calphostin C (CalC)

To confirm that c-*jun* induction occurs at the transcriptional level and examine the role of the AP1 site present in the c-*jun* promoter in the induction of c-*jun* by CalC, we performed transient transfections of c-*jun* promoter-CAT constructs harboring either (WT) AP1 regulatory element sites or mutant AP1 sites. CalC (250 nmol/L) induced a marked increase in CAT activity (i.e., promoter activation) with wt AP1 c-*jun* promoter-CAT plasmids, but the response was completely abrogated when constructs where the AP1 site was mutated were used (Fig. 4). PMA (300 nmol/L) caused a somewhat

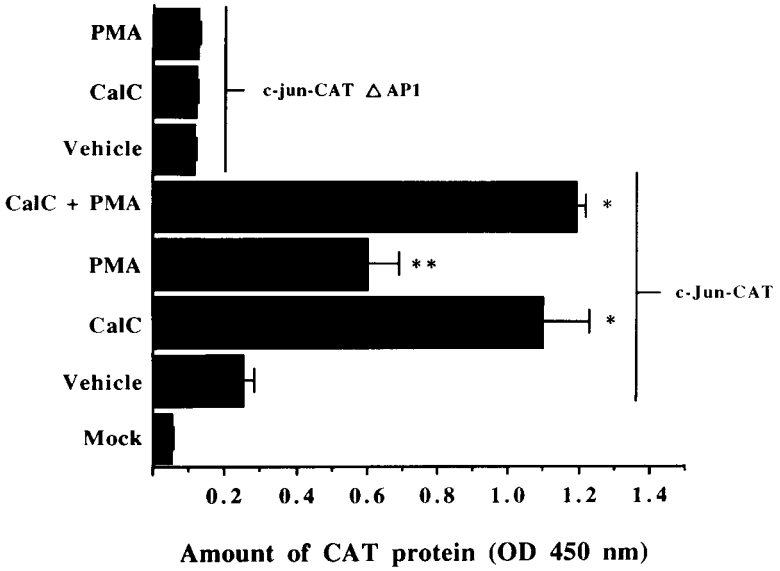

Fig. 4. Induction of c-jun promoter activity by CalC and PMA. Cells were co-transfected by the calcium phosphate precipitation method with 0.5 μg each of a plasmid expressing β-galactosidase (control of transfection efficiency) and the c-*jun*-CAT/ΔAP-1 *jun*-CAT reporter plasmids (2 μg each). Cells were activated with 250 nmol/L of CalC or 300 nmol/L of PMA for 20 h. Cells were then lysed in buffer containing Triton X-100 and the lysates assayed for β-galactosidase (colorimetry) and CAT activity by enzyme-linked immunosorbent assay (ELISA). The c-*jun*-CAT reporter plasmid was constructed by inserting a −79/+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 CalC and PMA. Values represent the amount (in OD units) of CAT protein in cell extracts divided by β-galactosidase activity (OD units) and are the means of three determinations in duplicate. Intra- and interassay coefficients of variation reached a maximum of 9% and 16%, respectively. Probability values (*P*) from Student's *t*-test: vehicle vs 250 nmol/L of CalC and 250 nmol/L of CalC + 300 nmol/L of PMA (*) was <0.001. **Vehicle vs 300 nmol/L of PMA, $P < 0.01$.

smaller increase in the level of CAT activity, and the increase was not abrogated by co-incubation with CalC (Fig. 4).

Calphostin C Inhibits MAP Kinase (p42/44) Activity but Stimulates JNK/SAPK

In order to explore the possibility that CalC may stimulate non-PKC signaling pathways associated with AP1 activation (directly or indirectly), we examined the effects of the inhibitor on two distinct proline-directed MAPK cascades: the MAP kinase pathway and JNK/SAPK pathway. To do so, we measured the activity of the terminal kinases (not withstanding the MAPKAP-K1 family) in each of the cascades, MAP kinase p42/44, also known as ERK1 and ERK2, respectively, and JNK/SAPK. As shown in Figure 5, CalC (250 nmol/L) inhibited in a time-dependent fashion the phosphorylation of Elk-1, a specific substrate of MAP kinase. Under the same experimental conditions, however, CalC increased the specific phosphorylation of c-Jun by JNK/SAPK (Fig. 5).

DISCUSSION

We recently identified some potential signaling pathways involved in the regulation of COX-2 gene expression in phenotypically stable human chondrocytes [Miller et al., 1998]. This was achieved with the use of two specific serine/threonine phosphatase (type 1 and 2A) inhibitors, namely okadaic acid (OKA) and calyculin A, which provided a cellular environment favoring an increased level of protein phosphorylation. One major MAP kinase cascade, the Raf/MEKK/MEK/ERK pathway, was inhibited by treatment with the phosphatase inhibitors, whereas another, the MEKK1/JNKK/JNK/SAPK pathway, was fully activated. In addition, PKA activity was stimulated, which was probably responsible for the Raf/MEKK/MEK/ERK pathway shutdown, as PKA inhibits Raf kinase activity [Cook and McCormick, 1993]. Two major transcription factor families were activated as a result of the activation of MEKK1/JNKK/JNK/SAPK and PKA pathways, namely AP1 and CREB/ATF, respectively. These latter

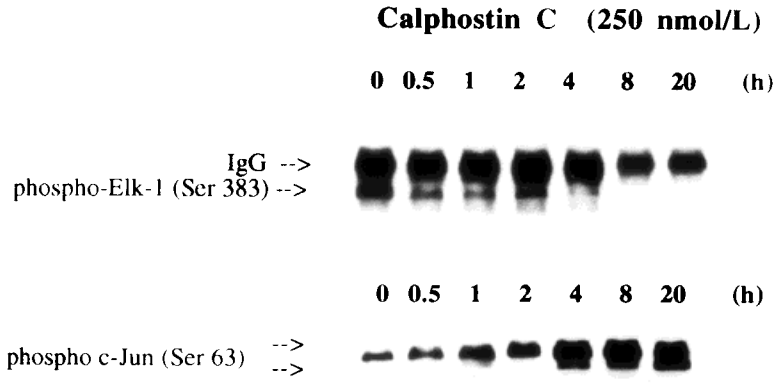

Fig. 5. Time course of CalC inhibition on mitogen-activated protein kinase (MAPK p42/44/ERK1,2) and stimulation of c-Jun N-terminal stress-activated protein kinase (JNK/SAPK). Serum-starved confluent quiescent chondrocytes were incubated with 250 nmol/L of CalC for 0, 0.5, 1, 2, 4, 8, and 20 h. Cellular proteins were extracted into RIPA buffer (plus phosphatase and protease inhibitors) and analyzed for MAPK (p42/44)/ERK1,2 and JNK/SAPK activity as described under Materials and Methods. Specific substrates were an Elk-1 fusion protein phosphorylated at ser 383 by ERKs and a 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-phosphosubstrate antibodies.

transacting factors play a pivotal role in the transactivation of the COX-2 gene in human chondrocytes by binding to the CRE in the promoter region [Xie and Herschman, 1995; Inoue et al., 1994]. Interestingly, PKC was not activated by OKA treatment; at first this was puzzling because PMA, a potent activator of PKC, strongly increases AP1 and COX-2 synthesis. In order to resolve this conundrum, we designed the present set of experiments to elucidate the role of PKC with the use of CalC, a specific inhibitor of the enzyme that binds not to the catalytic region, as most inhibitors do, but to the regulatory domain thereby increasing specificity immeasureably [Kobayashi et al., 1989; Bruns et al., 1991]. To our surprise, CalC mimicked the action of PMA in terms of AP1 synthesis while inhibiting the translocation of the principal chondrocyte PKC isoenzyme, PKC-α. Furthermore, CalC could induce transactivation through an AP1 site in the c-*jun* promoter. Although CalC has been shown to induce c-*jun* and c-*fos* mRNA in other cell types [Gamou and Shimizu, 1994; Freemerman et al., 1996], to our knowledge this is the first demonstration that the inhibitor can induce AP1 transactivation.

We interpreted our results to mean that CalC acts on signaling pathways independent of PKC to induce AP1. Recent evidence suggests that CalC can activate glycogen synthase kinase-3 (GSK-3) in A431 cells at concentrations that inhibit PKC, but it is inhibitory at higher concentrations [Lee and Yang, 1996]. The latter data provided us with interesting possibilities, since GSK-3 can indeed phosphorylate c-Jun [Boyle et al., 1991]. However, the kinase phosphorylates a threonine residue (239) in a domain of c-Jun that inhibits its ability to transactivate target promoters [Boyle et al., 1991]. Furthermore, GSK-3 is cytoplasmic, making it an unlikely candidate for a physiological nuclear c-Jun protein kinase [Hunter and Karin, 1992]. By contrast, coexpression of GSK-3α or β with c-Jun decreases its ability to transactivate an AP1-dependent reporter gene [de Groot et al., 1992], suggesting that GSK-3 may play a role in regulating c-Jun in vivo, although whether this occurs via direct c-Jun phosphorylation is unclear.

Some of the most important kinases that regulate AP1 DNA binding and transactivating ability are the MAP kinases, specifically JNK/SAPK, FRK, and possibly ERK1,2 [Karin, 1995]. These kinases are capable of phosphorylating c-Jun and family members at the correct Ser residue(s) in the transactivation domain of the protein [Kallunki et al., 1996]. The activity of these latter kinases is controlled by upstream kinases such as MEKK and JNNK/SEK [Cobb and Goldsmith, 1995]. It would appear that CalC increases AP1 DNA binding and transactivating ability by stimulating JNK/SAPK activity; ERK1 and 2 were inhibited by CalC under our experimental conditions and thus can be ruled out as mediators of AP1 activation. c-*fos* mRNA was induced by CalC and may be the major proto-oncogene stimulated, although

c-Fos is a poor substrate for JNK/SAPK [Deng and Karin, 1994]. As the inhibitor has the ability to activate proline-directed kinases, it is possible that FRK would also be stimulated in tandem with JNK/SAPK, although we have previously shown that the cellular levels of FRK in human chondrocytes are quite low (unpublished observations).

Our results suggest that CalC increases c-*jun* promoter activity though a TRE site, as a mutation in the sequence completely abrogated the response (in addition to evidence cited above). The TRE site in the c-*jun* promoter differs by 1 base pair (bp) from the consensus TRE and, although it has a high affinity for conventional AP-1 dimers, c-Jun/ATF2 dimers are bound more efficiently [Angel et al., 1987; Gupta et al., 1995]. We have no evidence that CalC can stimulate the synthesis and phosphorylation of ATF family members and, in fact, preliminary data in our laboratory seem to suggest an inhibitory pattern with respect to binding to ^{32}P-labeled ATF oligo, at least on a short-term basis (unpublished observations). However, conventional AP1 complexes can still potently transactivate through the TRE site in a manner quite similar to that exhibited by c-Jun/ATF2, and this fact may explain our results with CalC on c-*jun* promoter activation.

Calphostin C was classified as a specific PKC inhibitor, based on in vitro studies in which the inhibitor was reacted with purified preparations of various conventional isoforms of PKC [Kobayashi et al., 1989]. It was subsequently shown that photoactivation was essentially for maximum inhibitory activity [Bruns et al., 1991]. However, there were reports that CalC was not active with intact cells in culture [Gamou and Shimizu, 1994], and many of its effects were attributable to cytotoxicity. We have shown that CalC inhibits PKC activity both in vitro and in vivo [Di Battista et al., 1996] and, in this present study, we show that the inhibitor blocks cytoplasmic to membrane translocation of PKC-α in human chondrocytes in culture. Although we have identified another PKC-βI isoform expressed in chondrocytes, we have no evidence of its contribution to cellular PKC activity or whether it is sensitive to CalC.

The use of CalC with cells in culture has led to observations of cell toxicity. This may be not surprising since, in the presence of light, the molecule reacts with molecular oxygen to form free radicals. This can lead to membrane destruction and cell death. Other studies have demonstrated an effect of CalC akin to apoptosis, particularly in human glioma cells [Ikemoto et al., 1995; Freemerman et al., 1996]. Ikemoto et al. [1995] showed that the inhibitor (100 nmol/L) blocked PKC activity within 2–8 h and then stimulated Ca^{2+}/Mg^{2+}-dependent endonuclease activity after 16–24 h, a harbinger of DNA fragmentation and apoptosis. Furthermore, there was a downregulation of the proto-oncogene Bcl-2 (anti-apoptotic) expression and synthesis before cell death. Our cultures of human chondrocytes are more resistant to CalC than are glioma cells to the extent that, even at concentrations of ≤600 nmol/L, we observed no cellular toxicity. A preliminary check of cell extracts of human chondrocytes showed that (1) the cells produce relatively large amounts of Bcl-2, and (2) the levels are unaffected by treatment with CalC (John Di Battista, unpublished observations). Furthermore, we observed no proteolytic degradation of the nuclear lamellar protein, lamin B (unpublished observations), a target of the caspases, which is normally observed in cells undergoing apoptosis [Ucker et al., 1992].

From the pathophysiological point of view, the net effect of activation of the PKC-dependent signaling cascade on cartilage tissue homeostasis remains unclear. However, recent evidence obtained from animal models of osteoarthritis suggests that PKC activation is chondroprotective [Hamanishi et al., 1996; Kimura et al., 1994]. For example, synthesis of proteoglycans (PG), the basic building blocks of the cartilage matrix, is stimulated by phorbol esters. Transfection of chondrocytes with a PKC-α expression vector resulted in markedly elevated PG synthesis [Kimura et al., 1994]. Stimulation of keratan sulfate production by a histamine H1 agonist was inhibited by a PKC inhibitor and activated by phorbol ester [Fukuda et al., 1991]. It was recently observed that the PKC-α isoenzyme appears in larger quantities in osteoarthritic chondrocytes relative to normal chondrocytes [Hamanishi et al., 1996]. Interestingly, activation of PKC abrogated the interleukin-1 (IL-1)-induced stimulation of PG breakdown and inhibition of PG synthesis by cartilage in organ culture [Arner and Pratta, 1991].

In summary, CalC can induce AP1 synthesis and transactivation of AP1-sensitive target genes in human chondrocytes, probably through stimulation of JNK/SAPK kinase activity. With the demonstration of CalC-induced inhibition of the MAPK pathway, our results add to a growing list of signaling cascades affected by CalC that are PKC independent.

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