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Effects of Prostaglandins on Deoxyribonucleic Acid and Aggrecan Synthesis in the RCJ 3.1C5.18 Chondrocyte Cell Line: Role of Second Messengers*

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ABSTRACT

PGs play an important role in regulating articular chondrocyte function in both normal and pathological states. However, the mechanisms of the effects of PG on chondrocyte function remain undefined. We, therefore, examined the effects of PGE_1, PGE_2, and $PGF_{2\alpha}$ on second messenger generation in relation to DNA and aggrecan synthesis in the nontransformed rat RCJ 3.1C5.18 (RCJ) chondrocyte cell line. RCJ cells were grown under minimal attachment conditions on a composite collagen-agarose (0.15%/0.8%) gel to maintain a differentiated phenotype. PGE_1 and PGE_2 (0.001–100 μM) produced a similar dose-related increase in cAMP accumulation, with a maximal 8-fold increase over basal values, whereas $PGF_{2\alpha}$ produced a minimal 1.3-fold increase in cAMP levels only at 100 μM. On the other hand, both PGE_2 and $PGF_{2\alpha}$ raised the intracellular free calcium ($[Ca^{2+}]_i$) concentration, derived primarily from extracellular sources, whereas PGE_1 was without effect on $[Ca^{2+}]_i$. These three PGs also had divergent effects on DNA synthesis, as measured by [^{3}H]thymidine ([^{3}H]TdR) incorporation. $PGF_{2\alpha}$ (0.001–5 μM) produced a dose-related increase in [^{3}H]TdR incorporation, with a maximal 1.6-fold increase over baseline values at 5 μM and a slight decline to below maximal levels at 10 μM. PGE_2 exhibited a contrasting inverse biphasic response, with an initial small suppressive effect that was maximal at 0.1 μM and a secondary stimulatory phase producing a small increase over control values at 5 μM. PGE_1 had a uniformly suppressive effect, producing a 30% decrease at 10 μM. Despite the divergent effects of PGE_1, PGE_2, and $PGF_{2\alpha}$ on second messenger generation and DNA synthesis, all three PGs produced a dose-related stimulation of aggrecan synthesis. $PGF_{2\alpha}$ was the most potent, producing significant stimulation at 0.001 μM and a maximal 104% increase at 5 μM. PGE_1 and PGE_2 were approximately equipotent and approximately 60% as effective as $PGF_{2\alpha}$ in stimulating aggrecan synthesis. Northern analysis demonstrated that the effects of PG on aggrecan synthesis were not accompanied by changes in aggrecan core protein steady state messenger RNA levels. Thus, the effects of PG on aggrecan production in RCJ cells appear to be regulated at the posttranscriptional level. Forskolin and $(Bu)_2$cAMP mimicked the suppressive effects of PGE_1 on [^{3}H]TdR incorporation, as well as the stimulatory effect of PGE_1 on aggrecan synthesis. In addition, the phorbol ester 12-*O*-tetradecanoyl phorbol acetate mimicked $PGF_{2\alpha}$ stimulation of [^{3}H]TdR incorporation and aggrecan synthesis, and the effects of $PGF_{2\alpha}$ on these processes were blocked by protein kinase C inhibitors. Therefore, it appears that in mammalian chondrocytes, PGE_1 primarily activates the cAMP-protein kinase A second messenger system, $PGF_{2\alpha}$ affects primarily the Ca^{2+}-protein kinase C system, and PGE_2 activates both pathways. Moreover, PG posttranscriptional regulation of aggrecan synthesis in chondrocytes involves both the cAMP-protein kinase A and Ca^{2+}-protein kinase C second messenger systems. (*Endocrinology* **137:** 2208–2216, 1996)

PGs ARE IMPORTANT regulators of cellular function in a variety of tissues, including bone and cartilage (1). Chondrocytes play a central role in articular cartilage physiology, producing both the collagen and proteoglycan matrix and the various metalloproteinases that regulate articular cartilage status (2). Numerous reports suggest that PGs play a significant role in both normal articular cartilage metabolism and the pathogenesis of joint disorders (1, 2).

There is considerable evidence that PGs mediate many of the effects of polypeptide hormones, local growth factors, immune cytokines, estrogens, and mechanical forces on articular chondrocytes (2). In rheumatoid arthritis, much of the destruction of articular cartilage and periarticular bone is attributed to increased production of interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α, which stimulate increased metalloproteinase production and inhibit matrix protein synthesis by chondrocytes (2–4). Current evidence indicates that the various effects of these lymphokines are mediated via increased PG production (3–5). Moreover, increased metalloproteinase production occurs early in the course of osteoarthritis, and PGs appear to play a role in the pathogenesis of this disorder as well (6, 7).

Reported PG effects on chondrocyte metabolism include alterations of proteoglycan synthesis and stimulation of extracellular matrix degradation (2, 8–10). Chondrocytes produce a variety of PGs, including PGD_2, PGE_1, PGE_2, $PGF_{2\alpha}$, $PGF_{1\alpha}$, and thromboxane (8–10). PGE_1, PGE_2, and PGD_2 have biphasic effects on alkaline phosphatase activity and DNA

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synthesis in cultured chick chondrocytes (8), suggesting activation of multiple receptor systems. Moreover, PGE_1 and PGE_2 apparently have opposing effects on DNA synthesis in chick chondrocytes, suggesting that these two E series PGs may activate different combinations of receptors in these cells. This reported dichotomy of the effects of E series PGs has not been explored in mammalian chondrocytes. The autocrine regulation of chondrocyte function during development by PGs has not been well defined. However, endogenous PGE_2 production has been shown to correlate with the degree of differentiation and proteoglycan production in chick epiphyseal chondrocytes (8, 9). In addition, $PGF_{2\alpha}$, but not PGE_2, has been associated with accelerated bovine cartilage explant breakdown *in vitro* (10).

We have recently shown that 1) the effects of PGs on osteoblasts are mediated at least in part by second messengers generated by receptors linked to either the cAMP-protein kinase A (PKA) or Ca^{2+}-protein kinase C (PKC) systems; and 2) the actions of PGs on these two systems can have opposing effects on osteoblast function (11–14). In contrast, the second messenger mechanisms by which PGs and other factors regulate chondrocyte function are poorly defined. Both PGE_1 and PGE_2 have been shown to increase cAMP generation in synovial cells, chondrocytes, and other tissues (15, 16), and cAMP generation has been linked to stimulation of proteoglycan synthesis in rabbit articular chondrocytes (16). Moreover, PGE_1 blocks the progression of adjuvant-induced arthritis in rats, apparently in part via stimulation of the cAMP-PKA system (17, 18), whereas $PGF_{2\alpha}$ does not increase intracellular cAMP and does not ameliorate adjuvant arthritis (15). The effects of $PGF_{2\alpha}$ on second messengers in chondrocytes have not been otherwise examined.

Ca^{2+} also appears to play an important signaling role in chondrocytes. In embryonic chick chondrocytes, decreased ambient Ca^{2+} concentration and cartilage-derived growth factor produce a decrease in the intracellular free calcium concentration ($[Ca^{2+}]_i$), in association with decreased proliferation and increased proteoglycan synthesis (19, 20). In contrast, serum stimulation of mitogenesis in pig chondrocytes is associated with a rise in $[Ca^{2+}]_i$ (21). In addition, phorbol ester stimulation of PKC activity in chick chondrocytes directly alters type II collagen gene expression (22, 23). Thus, it appears that the PKA and PKC-$[Ca^{2+}]_i$ second messenger systems play a major role in regulating chondrocyte proliferation and function, and that reported differences between the actions of E and F series PGs could potentially be attributable to their varying potencies in activating these two systems. However, PG regulation of second messenger systems in relation to effects on cellular function in chondrocytes has not been systematically examined.

Recently, the RCJ 3.1C5.18 (RCJ) nontransformed rat chondrocyte line has been subcloned from a pluripotential mesodermal stem cell line derived from neonatal rat calvaria (24, 25). We have recently demonstrated that this cell line exhibits a highly chondrocytic phenotype when grown under minimal attachment conditions (26, 27). Therefore, in the present study we have employed the RCJ cell line to examine the effects of PG on chondrocyte function in relation to second messenger generation and action.

Materials and Methods

Materials

Culture media, hormones, PGs, purified type VII collagenase, and all test reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Rat PTH-(1–34) amide (PTH) was obtained from Peninsula Laboratories (Belmont, CA), and [^{125}I]cAMP RIA kits were purchased from Incstar (Stillwater, MN). RCJ 3.1C5.18 cells were generously donated by Dr. Jane E. Aubin (University of Toronto, Toronto, Canada).

Cell culture

RCJ cells in maintenance culture were grown in αMEM supplemented with 15% FCS (HyClone, Logan UT), 10 nM dexamethasone, and 50 μg/ml ascorbic acid to maintain maximal phenotypic stability (24, 25) and were used before passage 10. For experimental use, cells were switched to αMEM, supplemented with 5% FCS, 1 nM dexamethasone, and 50 μg/ml ascorbic acid in a humidified 95% air-5% CO_2 atmosphere at 37 C, 24 h before initiating studies. Omitting dexamethasone from the culture medium did not significantly alter experimental results (data not shown). For passaging or replating, cells cultured in monolayer on plastic plates or dishes were released as a single cell suspension by washing twice with calcium- and magnesium-free PBS, incubating with 0.2% collagenase-0.05% hyaluronidase in PBS for 15 min at 37 C, and then treating briefly with 2.5 mM EDTA followed by 0.0004% trypsin (Sigma Chemical Co., St. Louis, MO) at 37 C for 1 min. PGs were dissolved in absolute ethanol and diluted in culture medium, with equal amounts of vehicle added to all cultures. Ethanol concentrations never exceeded 0.05%. PGs were added to the cultures at the time of a medium change. Cell counts were performed by hemocytometer on cells released by trypsin-EDTA from parallel treated cultures, and all results were normalized for cell number.

To produce a minimum contact cell growth substrate, plastic culture plates were coated with a composite collagen-agarose (CAG) substrate composed of 0.8% low melt preparative grade agarose (Bio-Rad, Hercules, CA) agarose and 1.5 mg/ml neutralized rat tail type I collagen (Collagen Corp., Santa Clara, CA) in DMEM, as previously described (26, 27). One to 3×10^6 cells in 2–4 ml growth medium were then plated on the surface of the solidified CAG substrate and allowed to attach for 48 h before the media were changed. Cells grown on CAG substrate were released by treatment with 0.4% collagenase and 0.1% hyaluronidase in PBS at 37 C for 30 min, without trypsinization. Cells differentiated in agarose culture or by growth on CAG substrate were replated in monolayer on a plastic surface for 16 h before use, according to techniques routinely used in primary chondrocyte cultures (28, 29). RCJ cells replated in this manner have been shown to maintain their differentiated chondrocytic phenotype, as determined by collagen subtype synthesis for at least 48 h after return to monolayer culture on a plastic surface (26, 27).

Two-dimensional (2-D) cyanogen bromide peptide mapping

Minor modifications of our previously described techniques (26–28) were employed. Cells were switched to medium containing 1% FCS, β-aminoproprionitrile (62.5 μg/ml), and ascorbic acid (25 μg/ml) in 25-cm^2 flasks and labeled for 24 h with 100 μCi [^{3}H]proline. Whole cultures were adjusted to 0.5 M acetic acid and treated with 250 μg/ml pepsin (Sigma) for 24 h. Radioactive collagen was then isolated by sequential neutral NaCl precipitation, cleaved under N_2 with a 4-fold excess (wt/wt) of cyanogen bromide (10 mg/ml in 70% formic acid) at 30 C for 4 h, and subjected to 2-D electrophoresis. Cleavage fragments on the 2-D maps were then quantified and analyzed by image analysis.

DNA synthesis

Cells were incubated with test agents or vehicle in serum-free medium supplemented with 0.1% BSA in 24-multiwell plates at 37 C for 24 h. [^{3}H]Thymidine (2 μCi/ml; [^{3}H]TdR) with 10 μM unlabeled TdR was added for the last 4 h. Incubations were terminated by washing with ice-cold PBS, and [^{3}H]TdR incorporation into TCA-insoluble DNA was determined as previously described (30).

Proteoglycan synthesis

Modifications of previously described techniques were used (28). Cells grown in 1.0 ml medium in 12-well plates were labeled with 10 μCi carrier-free $^{35}SO_4$ (ICN, Irvine, CA) and incubated in the presence of test agents for 24 h Incorporation was terminated by the addition of 150 μl dissolution solution, composed of 383 mM 3-[*N*-morpholino]propanesulfonic acid (pH 6.5), 0.77% Triton X-100, 0.383% sodium azide, and pronase (Calbiochem, La Jolla, CA), and cells were incubated overnight at 55 C in sealed plates. The digests were treated with 5 mM EDTA-0.05% sodium azide at room temperature for 1 h, and 150-μl aliquots were spotted on 13-mm glass fiber filters (Millipore Corp., Bedford, MA). The filters were then dried at 55 C for 1 h, extracted 3 × for 1 h in 0.2% cetyltrimethylammonium bromide (Sigma)-50 mM EDTA (pH 6.0), and then subjected to liquid scintillation counting. The proportion of total $^{35}SO_4$-labeled proteoglycan corresponding to aggrecan was determined by extraction of whole cultures with 4 M guanidium hydrochloride, 10 mM EDTA, and 50 mM sodium acetate (pH 6.5), followed by sequential size exclusion separation over Sephadex G-25 (PD-10), then 0.8 × 30-cm Sepharose CI4B columns eluted with the extraction buffer, as previously described (31).

Northern hybridization

Total cellular RNA was prepared from cells using the single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction (32). Equal amounts of RNA (15 μg/lane) were fractionated on 1.5% agarose-formaldehyde gels and transferred to UV-Duralone nylon membrane (Stratagene, La Jolla, CA), followed by UV cross-linking treatment. Prehybridization and hybridization were performed at 68 C for 2 h using Quikhyb (Stratagene) hybridization solution and ^{32}P-labeled complementary DNA (cDNA) probes. The membranes were washed twice at room temperature with 1 × SSC (0.15 M NaCl, 0.015 M Na Citrate, pH 7.0)-0.1% SDS for 2 min, then once at 37 C with 0.1 × SSC-0.1% SDS for 15 min, and exposed to x-ray film (Kodak XAR-5, Eastman Kodak, Rochester, NY)).

cDNA probes

The aggrecan core protein cDNA probe (33) was kindly provided by Dr. Y. Yamada (National Institute of Dental Research, Bethesda, MD), and the glyceraldehyde-3-phosphate dehydrogenase probe was a 1.3-kb clone encoding rat glyceraldehyde 3-phosphate dehydrogenase (34).

$[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was measured by our established fura-2 fluorescent probe techniques (30, 35), using a Photon Technologies Deltascan I dual wavelength fluorescence spectrophotometer with image analyzer (Photon Technology International, South Brunswick, NJ). Measurements were performed in cells attached to coverslips fitted onto the thermostatted stage of a Nikon Diaphot inverted microscope (Nikon Corp., Melville, NY). Groups of 20–30 cells were viewed with a ×40 fluorescence objective, excited alternately at 340 and 380 nm, and emission was measured at 505 nm.

Intracellular cAMP measurement

Cells in 24-well plates were preincubated at 25 C for 10 min in serum-free medium supplemented with 0.1% BSA and containing 0.5 mM 3-isobutyl-1-methylxanthine, then exposed to test agents for an additional 5 min. The cells were washed three times with cold PBS, and cAMP was extracted with 1 ml cold 95% ethanol-20 mM HCl at −20 C overnight. Extracts were evaporated under nitrogen gas and reconstituted in sodium acetate buffer (pH 6.2) for RIA, as previously described (30, 35).

Statistical analysis

Values represent the mean ± SEM for the indicated number of replicate samples, representative of three or more experiments. The statistical significance of differences between group means was determined by Student's *t* test after ANOVA testing.

Results

Chondrocytic phenotype expression

In accord with our previous observations (26, 27), RCJ cells maintained in minimum contact CAG culture for 7 days and then returned to monolayer culture for 16 h before use exhibited a highly differentiated chondrocytic phenotype, as demonstrated by rounded chondrocytic morphology, high levels of aggrecan synthesis and messenger RNA (mRNA) expression, and strong expression of link protein mRNA (data not shown). The differentiated chondrocytic phenotype was further demonstrated by quantitative 2-D gel electrophoresis demonstration of 77.1% type II collagen and only 9.2% type I collagen synthesis after 7 days in CAG culture (mean of two experiments). Type I and II collagen mRNA levels paralleled collagen protein production (data not shown). Cells prepared in this manner were used for all experiments.

Effects of PGs on intracellular cAMP and $[Ca^{2+}]_i$

Both PGE_1 and PGE_2 markedly stimulated cAMP generation in RCJ cells, and the two E series PGs were approximately equipotent in this regard (Fig. 1). For both PGs, significant stimulation occurred at 1 nM, the EC_{50} was approximately 50 nM, and a maximal 9-fold increase over basal values was produced by 100-μM concentrations. In contrast, $PGF_{2\alpha}$ was virtually without effect on cAMP generation, producing only a marginal increase at the highest concentration tested (100 μM). Maximally effective concentrations of PGE_1 and PGE_2 were not additive in stimulating cAMP accumulation, implying that the two PGs share a common receptor and/or pool of adenylate cyclase. Coaddition of 100 μM $PGF_{2\alpha}$ with 100 μM PGE_1 or PGE_2 did not influence the effects of E series PGs on cAMP stimulation (Table 1).

Basal $[Ca^{2+}]_i$ in RCJ cells was 128 ± 6 nM (mean ± SE for eight determinations). Relative to effects on cAMP generation, a striking difference was observed in the specificity of the effects of PG on $[Ca^{2+}]_i$. $PGF_{2\alpha}$ produced a rapid increase in $[Ca^{2+}]_i$, with the rapid phase increase maximal at about 1 min (Fig. 2A). Thereafter, $[Ca^{2+}]_i$ remained persistently elevated for at least 10 min. PGE_2 produced an identical $[Ca^{2+}]_i$ response (data not shown). In the absence of extracellular Ca^{2+}, both rapid and sustained phases of the PG-induced increase in $[Ca^{2+}]_i$ were abolished, indicating that the Ca^{2+} was derived from an extracellular source (Fig. 2B). $PGF_{2\alpha}$ was by far the most potent PG tested in terms of its effect on $[Ca^{2+}]_i$. The mean maximal increase in $[Ca^{2+}]_i$ over baseline produced by $PGF_{2\alpha}$ was 270 ± 30 nM (n = 8) *vs.* 175 ± 15 nM for PGE_2 (n = 8; $P < 0.001$). As shown in Fig. 3, PGE_1 was without significant effect on $[Ca^{2+}]_i$ at the highest concentration tested (100 μM). $PGF_{2\alpha}$ produced a significant effect on $[Ca^{2+}]_i$ at 0.001 μM, and the maximal effect occurred at 1 μM, with an EC_{50} of 0.015 μM. In contrast, the stimulatory effect of PGE_2 on $[Ca^{2+}]_i$ was not detectable below 0.1 μM, a concentration 10-fold higher than the minimal effective concentration for $PGF_{2\alpha}$ (Fig. 3).

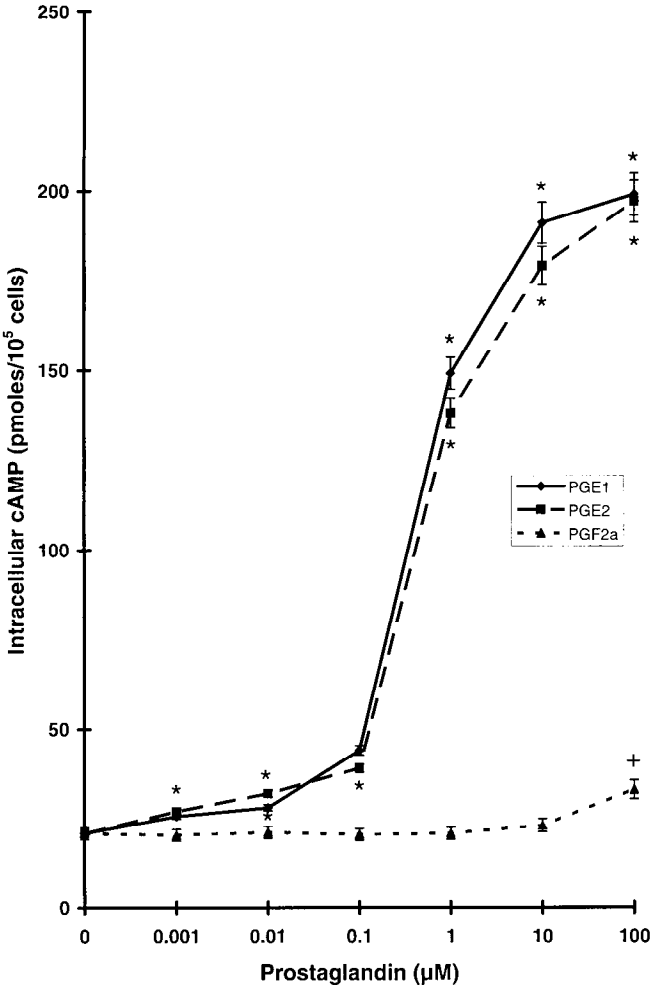

FIG. 1. Effects of PGs on intracellular cAMP accumulation in RCJ cells. Incubations were performed in the presence of 0.5 mM 3-isobutyl-1-methylxanthine, and cAMP was determined by RIA, as described in *Materials and Methods*. Each value represents the mean ± SEM of five replicates. +, $P < 0.02$; *, $P < 0.001$ (*vs.* control).

TABLE 1. Effects of PGs, alone and in combination, on intracellular cAMP accumulation

Treatment	Intracellular cAMP (nmol/10^6 cells)
Control	18 ± 2
PGE_1	600 ± 54[a]
PGE_2	580 ± 38[a]
$PGF_{2\alpha}$	27 ± 4
PGE_1 + PGE_2	625 ± 43[a]
PGE_1 + $PGF_{2\alpha}$	594 ± 51[a]
PGE_2 + $PGF_{2\alpha}$	578 ± 47[a]

All PGs were present at 100 μM, and cells were exposed to agents for 5 min in the presence of 0.2 mM IBMX. Values represent the mean ± SEM for five determinations. Results are representative of three similar experiments. No significant additivity of PG effects was observed.

[a] $P < 0.001$ *vs.* control.

Effects of PGs on DNA and aggrecan synthesis

The effects of PGs on DNA synthesis also varied markedly (Fig. 4). $PGF_{2\alpha}$ was primarily stimulatory, with a minimally effective concentration of 0.001 μM, an EC_{50} of 0.094 μM, and a maximal 1.6-fold stimulatory effect ($P < 0.001$) at 1 μM. At the highest concentration tested (10 μM), the stimulatory effect of $PGF_{2\alpha}$ declined slightly below peak levels. PGE_2 produced a contrasting inverse biphasic effect, with an initial small suppressive effect, a maximal 26% decrease below control values at 0.1 μM ($P < 0.01$), and a subsequent stimulatory phase, producing a 14% increase over control values ($P < 0.01$) at 10 μM. PGE_1 exhibited a third type of response pattern, with a mild uniformly suppressive effect significant at concentrations of 0.01 μM and above and a maximum 30% decrease in DNA synthesis ($P < 0.001$) at 10 μM.

Finally, despite the divergent effects of PGE_1, PGE_2, and $PGF_{2\alpha}$ on second messenger generation and DNA synthesis,

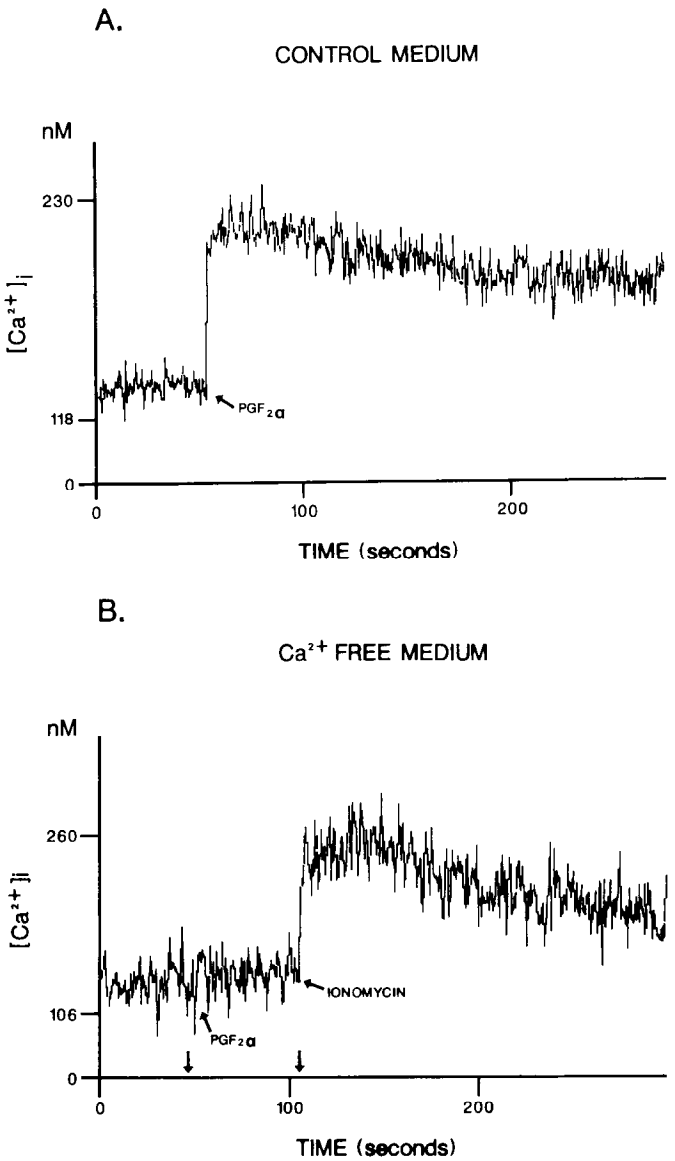

FIG. 2. $[Ca^{2+}]_i$ response to $PGF_{2\alpha}$ stimulation in RCJ cells. Measurements were performed in cells attached overnight to glass coverslips as described in *Materials and Methods*, and $PGF_{2\alpha}$ (5 μM) or ionomycin (10 nm) was added at the time points indicated by the *vertical arrows*. Values were determined in medium containing 1.2 mM Ca^{2+} (A) or in medium containing 1 mM EGTA and no added Ca^{2+} (B). The positive response to added ionomycin in the cell incubated in the absence of extracellular Ca^{2+} indicates the presence of releasable intracellular Ca^{2+} stores.

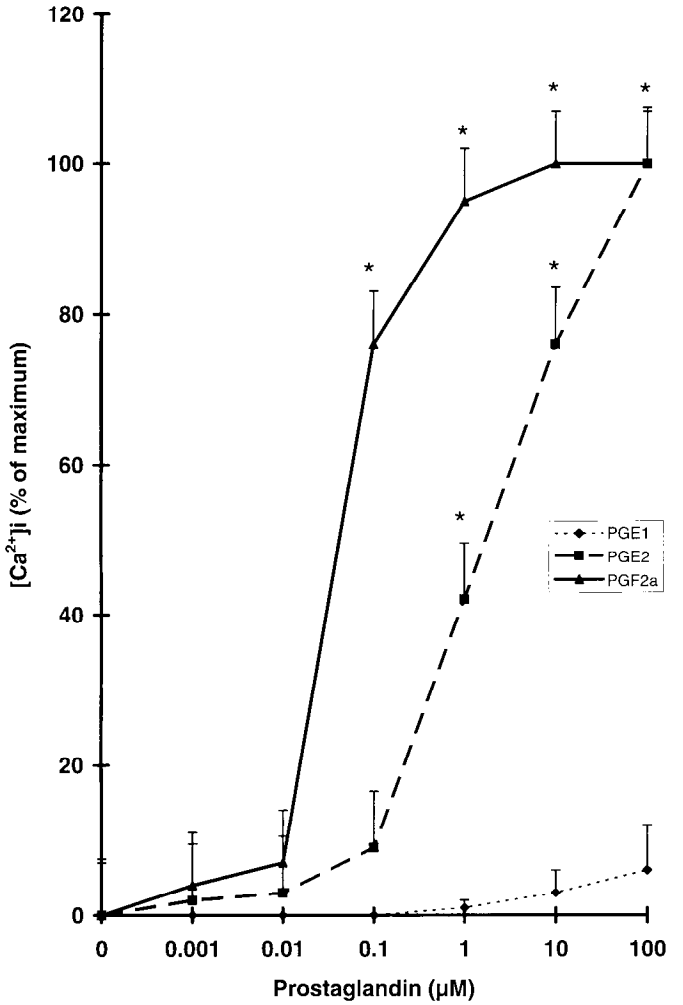

FIG. 3. Dose-response of the effects of PG on $[Ca^{2+}]_i$ in RCJ cells. Incubations were performed in cells attached to glass coverslips, and $[Ca^{2+}]_i$ was determined as described in *Materials and Methods*. Each value represents the mean ± SEM of five replicates. +, $P < 0.02$; *, $P < 0.001$ (*vs.* control).

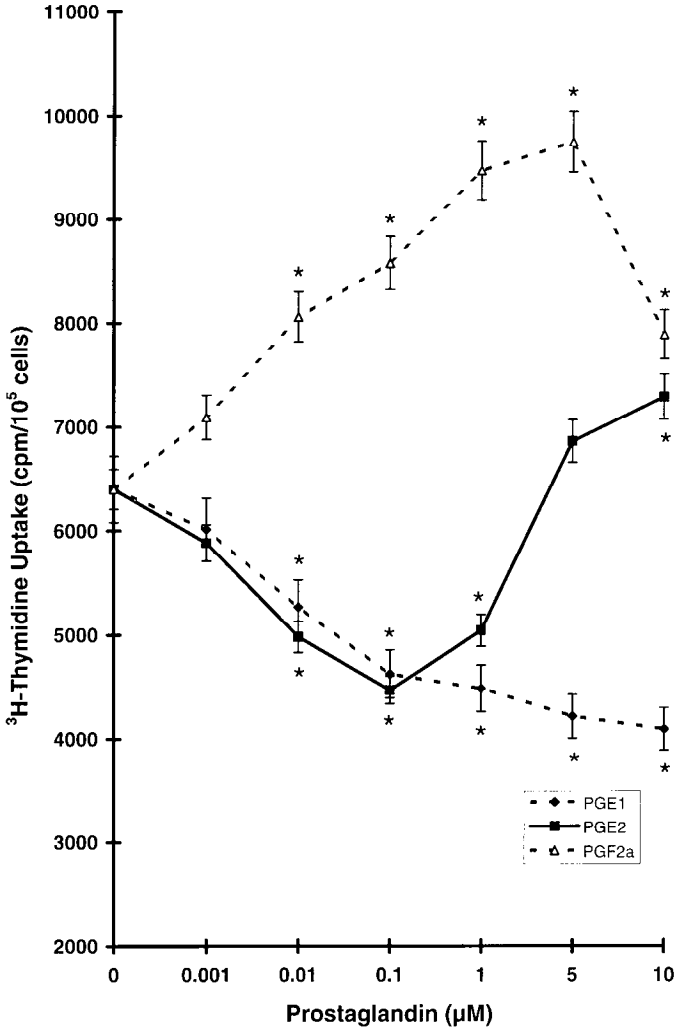

FIG. 4. Effects of PG on DNA synthesis in RCJ cells. Incubations were performed, and DNA synthesis was determined by [^{3}H]TdR incorporation as described in *Materials and Methods*. Each value represents the mean ± SEM of five replicates. +, $P < 0.02$; *, $P < 0.001$ (*vs.* control).

all three PGs produced a dose-related stimulation of aggrecan synthesis (Fig. 5). $PGF_{2\alpha}$ was the most potent in this regard, producing a 28% increase over control values ($P < 0.01$) at 0.001 μM and a maximal 104% increase ($P < 0.001$) at 5 μM, with an EC_{50} of 0.016 μM. PGE_2 and PGE_1 produced a similar, less marked, pattern of stimulation of aggrecan synthesis. The effects of the two E series PGs were not apparent below 0.01 μM, and both produced a maximal approximately 60% increase at 10 μM. Sequential Sephadex G-25 and Sepharose Cl4B size separation column profiles demonstrated that greater than 95% of proteoglycan synthesis in control cultures, as measured by ^{35}S incorporation, corresponded to aggrecan by standard mol wt criteria. This proportion was not significantly altered by any of the PGs tested. Northern analysis of the effects of PGs on aggrecan core protein mRNA levels demonstrated that there were no significant changes produced by any PG at 2 μM, the maximally effective concentration for all three PGs (Fig. 6). Thus, the stimulatory effects of PGs on aggrecan production in RCJ cells appear to be regulated almost exclusively at the post-transcriptional level. The addition of 1 μM indomethacin to the culture medium for 24 h before treatment with PGs did not significantly alter second messenger or functional responses to the PGs tested, indicating that endogenous PG production did not influence the responses to the PGs examined in these studies.

To examine the role of PG-stimulated second messenger generation on chondrocyte function, we compared the effects of forskolin (FSK) and $(Bu)_2$cAMP, two potent stimulators of intracellular cAMP levels, with the effects of PKC stimulation produced by 12-*O*-tetradecanoyl phorbol acetate (TPA) on DNA and aggrecan synthesis in RCJ cells. Both FSK and $(Bu)_2$cAMP inhibited DNA synthesis in a dose-related fashion, whereas TPA at low concentrations was mildly stimulatory (Table 2). All three agents produced a dose-related stimulation of PG production, suggesting that the PKA and PKC systems both mediate positive regulatory effects on aggrecan expression. In addition, the stimulatory effects of 5 μM $PGF_{2\alpha}$ and PGE_2 on DNA and aggrecan synthesis were

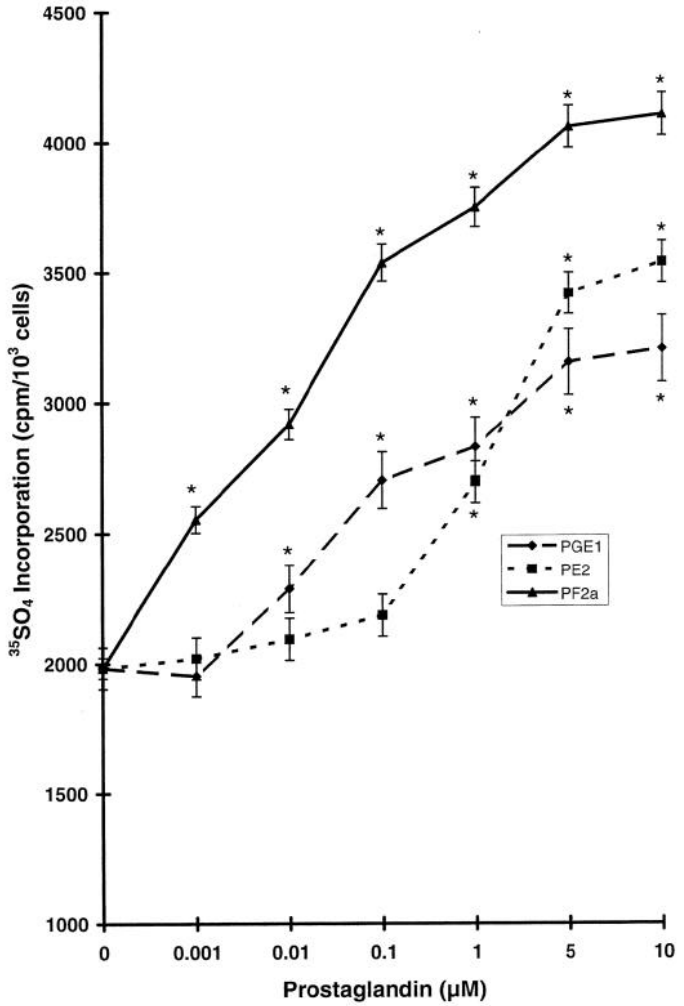

FIG. 5. Effects of PG on aggrecan synthesis, as determined by ^{35}S incorporation in RCJ cells. Incubations were performed, and aggrecan synthesis was determined by ^{35}S incorporation into cetyltrimethylammonium bromide-insoluble material and sizing column separation of cell extracts as described in *Materials and Methods*. Each value represents the mean ± SEM of five replicates. +, $P < 0.02$; *, $P < 0.001$ (*vs.* control).

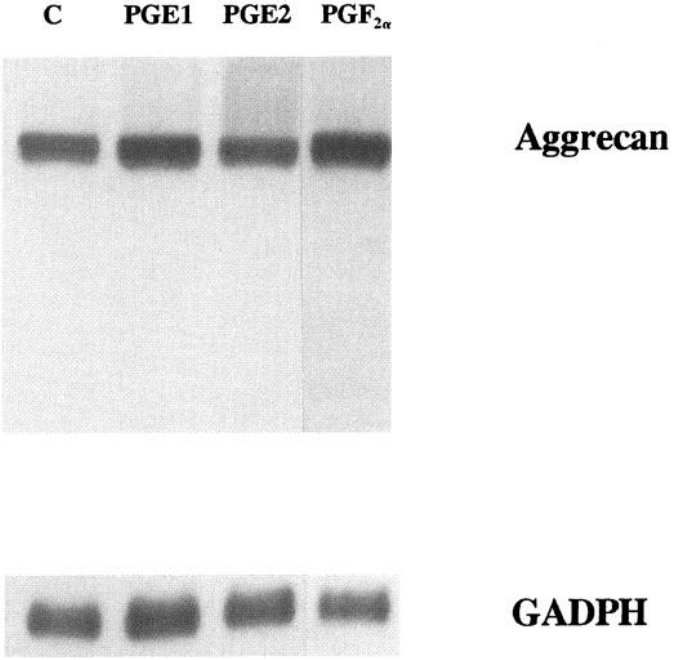

FIG. 6. Effects of PG on aggrecan core protein steady state mRNA levels in RCJ cells. mRNA levels were determined in RNA extracts obtained after 16 h of exposure to test PGs. Similar results were obtained after 4- and 24-h exposures to PGs (not shown).

TABLE 2. Effects of forskolin (FSK), $(Bu)_2$cAMP, and TPA on DNA and proteoglycan synthesis in RCJ cells

	[^{3}H]TdR (cpm/10^6 cells)	^{35}S incorporation (cpm/10^6 cells)
Control	6043 ± 184	2290 ± 135
FSK (0.1 mM)	5817 ± 199	2418 ± 166
FSK (1.0 mM)	5090 ± 208[a]	3402 ± 170[a]
FSK (10 mM)	5090 ± 208[a]	3402 ± 170[a]
$(Bu)_2$cAMP (5 mM)	5688 ± 220	2511 ± 144
$(Bu)_2$cAMP (50 mM)	4720 ± 201[a]	2947 ± 171[a]
$(Bu)_2$cAMP (500 mM)	4019 ± 217[a]	3690 ± 273[a]
Control	5808 ± 164	2384 ± 183
TPA (5 ng/ml)	6795 ± 213	2495 ± 216
TPA (10 ng/ml)	7368 ± 240[a]	2814 ± 199[a]
TPA (50 ng/ml)	8944 ± 291[a]	3218 ± 247[a]
TPA (100 ng/ml)	6830 ± 297[a]	2702 ± 221[a]

Incubations were performed for 24 h, as described in *Materials and Methods*, with the test reagents present for the entire period. [^{3}H]TdR was added 2 h before termination of the incubations, whereas ^{35}S was present during the entire 24 h. Incubations were terminated and individual determinations performed as described in *Materials and Methods*. Each value represents the mean ± SEM for six replicates. These results are representative of five separate experiments.

[a] $P < 0.01$ *vs.* control.

blocked by PKC inhibitors, suggesting that the PKC second messenger system is involved in these stimulatory effects (Table 3).

Discussion

The present study demonstrates that PGE_1, PGE_2, and $PGF_{2\alpha}$ all influence RCJ chondrocyte cellular activity, but produce strikingly different patterns of effect on RCJ cell second messenger generation and functional responses. With regard to effects on cAMP generation, PGE_1 and PGE_2 were both markedly stimulatory and approximately equipotent, with significant stimulation seen at 0.001 μM and above, and an EC_{50} of approximately 50 nM. Maximally effective concentrations of PGE_1 and PGE_2 were not additive with regard to stimulation of cAMP accumulation, suggesting that they share a common receptor and/or pool of adenylate cyclase. In contrast to the E series PGs, $PGF_{2\alpha}$ had only minimal effect on cAMP levels at the highest concentration tested (100 μM).

A quite different pattern was observed with regard to PG stimulation of $[Ca^{2+}]_i$. Basal $[Ca^{2+}]_i$ in RCJ chondrocytes was approximately 128 nM, similar to the resting values that we have previously observed in UMR-106-01 osteoblastic osteosarcoma cells and normal rat osteoblasts (11, 30, 35). $PGF_{2\alpha}$ was a highly potent stimulator of $[Ca^{2+}]_i$ in RCJ cells, with detectable effects at 0.001 μM and an EC_{50} of 15 nM. Although PGE_2 also stimulated $[Ca^{2+}]_i$, approximately 100-

TABLE 3. Effects of chelerythrine chloride (CC) and H7 on DNA and proteoglycan synthesis in RCJ cells

	[^{3}H]TdR (cpm/10^6 cells)	^{35}S incorporation (cpm/10^6 cells)
Control	5319 ± 206	2132 ± 190
CC (1 μM)	4990 ± 221	1714 ± 218
H7 (5 μM)	4809 ± 214	1893 ± 196
$PGF_{2\alpha}$ (5 μM)	8739 ± 361[a]	3718 ± 249[a]
$PGF_{2\alpha}$ + CC	5213 ± 224	2317 ± 220
$PGF_{2\alpha}$ + H7	4796 ± 216	2229 ± 190

Incubations were performed for 24 h, as described in *Materials and Methods*, and test reagents were present for the entire period. [^{3}H]TdR was added 2 h before termination of the incubations, whereas ^{35}S was present during the entire 24 h. Incubations were terminated and determinations performed as described in *Materials and Methods*. Each value represents the mean ± SEM for five replicates. These results are representative of three separate experiments.

[a] $P < 0.01$ *vs.* control.

fold higher concentrations of PGE_2 were required for a detectable effect. Moreover, the maximal increase in $[Ca^{2+}]_i$ produced by $PGF_{2\alpha}$ was 1.5-fold greater than that produced by PGE_2. In contrast to its potent effects on cAMP stimulation, PGE_1 had no significant effect on $[Ca^{2+}]_i$. Stimulation of $[Ca^{2+}]_i$ by $PGF_{2\alpha}$ and PGE_2 appeared to primarily involve Ca^{2+} entry from extracellular sources, as the stimulatory effect was abolished by the removal of extracellular Ca^{2+}. This implies that $PGF_{2\alpha}$ and PGE_2 produce plasma membrane calcium channel activation in chondrocytes, in parallel with our previous observations in osteoblasts (11).

Therefore, it appears that in RCJ chondrocytes, PGE_1 activates primarily the cAMP-PKA second messenger system, $PGF_{2\alpha}$ affects primarily the Ca^{2+}-PKC system, and PGE_2 activates both pathways. This pattern of PG second messenger generation resembles that previously observed in normal and transformed osteoblasts (11–13, 30, 35). Whether this represents a general PG second messenger response pattern in all cells of mesenchymal origin remains to be determined.

With regard to effects on cell function, PGE_1, PGE_2, and $PGF_{2\alpha}$ had markedly divergent effects on DNA synthesis. $PGF_{2\alpha}$ in concentrations from 0.001–5 μM was uniformly stimulatory, with an EC_{50} of approximately 20 nM, which was very close to its EC_{50} for stimulation of $[Ca^{2+}]_i$. However, the stimulatory effect of $PGF_{2\alpha}$ at 10 μM, the highest concentration tested, was consistently less than the maximal stimulation produced by 1–5 μM $PGF_{2\alpha}$. In contrast to the effects of $PGF_{2\alpha}$, PGE_1 was uniformly inhibitory. Thus, $PGF_{2\alpha}$, which appeared to solely activate the Ca^{2+}-PKC system in concentrations from 0.001–5 μM, had purely stimulatory effects on DNA synthesis over this concentration range, whereas PGE_1, which stimulated only cAMP generation, had uniformly inhibitory effects. This suggests that activation of the cAMP-PKA system suppresses DNA synthesis in chondrocytes, a hypothesis that is supported by the fact that both FSK and $(Bu)_2$cAMP had uniformly suppressive effects on [^{3}H]TdR incorporation. A similar inhibitory effect of cAMP-stimulating agents on DNA synthesis has been observed in osteoblasts (13). In addition, the stimulatory effect of $PGF_{2\alpha}$ on DNA synthesis was mimicked by low doses of TPA and blocked by PKC inhibitors, suggesting a role for the PKC system in stimulating DNA synthesis in these cells. Similar conclusions have been reached regarding the role of the PKC system in stimulating mitogenesis in osteoblasts and other cell types (11–14), although all cellular kinase inhibitor studies must be interpreted with caution because the specificity of these agents can vary considerably.

These observations, therefore, suggest that the biphasic effects of PGE_2 on DNA synthesis may be related to its ability to stimulate both the cAMP-PKA and Ca^{2+}-PKC second messenger pathways. Indeed, the stimulatory effect of PGE_2 on DNA synthesis occurred at concentrations just above its minimal effective concentration for detectable stimulation of $[Ca^{2+}]_i$ (0.1 μM), whereas the minimal effective concentration for PGE_2 stimulation of cAMP generation was 100-fold lower (0.001 μM). Similarly, $PGF_{2\alpha}$ at 10 μM, a concentration approximating that at which mild stimulation of cAMP generation was observed, produced less stimulation of DNA synthesis than that produced by lower concentrations that appeared to have no stimulatory effect on intracellular cAMP levels despite the fact that effects on $[Ca^{2+}]_i$ were undiminished.

In contrast to their divergent effects on DNA synthesis, PGE_1, PGE_2, and $PGF_{2\alpha}$ all had stimulatory effects on aggrecan synthesis. $PGF_{2\alpha}$ was approximately 1.7-fold more potent than PGE_1 and PGE_2 in this regard, but all three PGs produced monophasic dose-related response curves. As aggrecan core protein mRNA levels were not altered by any of the PGs tested, at concentrations at which significant effects on aggrecan production were observed, it appears that primarily posttranscriptional effects are involved in the stimulatory effects of PGs on aggrecan synthesis observed in the present study. This is in contrast to the significant role that transcriptional mechanisms appear to play in the stimulation of increased aggrecan production associated with surface contact-mediated enhanced differentiation in chondrocytes, including the RCJ cell line (26–28, 31). Although the standard ^{35}S incorporation assay for aggrecan synthesis used in the present studies does not dissociate effects on aggrecan protein synthesis from possible posttranslational alterations in sulfate incorporation, there appears to be a close correlation between these two processes (31, 36, 37). However, more precise delineation of the pre- and posttranscriptional mechanisms of the effects of PG on aggrecan core protein mRNA levels will require further detailed mRNA time-course and stability studies.

The consistently stimulatory effects of PGE_1, PGE_2, and $PGF_{2\alpha}$ on aggrecan synthesis stand in contrast to their divergent effects on second messenger generation and DNA synthesis. Among the possible explanations are that 1) these PGs generate an additional common second messenger not examined in these studies; and 2) the cAMP-PKA and Ca^{2+}-PKC systems both have stimulatory effects on aggrecan gene expression. With regard to the first possibility, to date there is no evidence that second messenger pathways other than those examined in the present studies have any significant role in the effects of PG on cellular function (1, 8, 11–14). With regard to the possibility that the PKA and Ca^{2+}-PKC pathways may both have stimulatory effects on aggrecan synthesis, it is well recognized that the regulation of aggrecan gene expression is complex and involves transcriptional, translational, and posttranslational mechanisms (36). Insulin-like growth factor I and other growth factors stimulate

aggrecan gene expression at both transcriptional and post-transcriptional levels (31), suggesting that the tyrosine kinase system may be involved in aggrecan core protein gene regulation (38). However, PG stimulation of the tyrosine kinase second messenger system has not been reported. As no PG effects on aggrecan core protein mRNA levels were observed in the present study, it must be concluded that under the conditions employed here, the effects of PG on aggrecan production were posttranscriptional. The fact that both forskolin and low doses of PMA had stimulatory effects on aggrecan protein production in the present study suggests that both the PKA and PKC systems may have posttranscriptional effects on aggrecan protein synthesis in mammalian chondrocytes. Stimulation of aggrecan synthesis by low doses of PMA has also been noted in primary cultures of bovine articular chondrocytes (39).

The potential physiological relevance of these observations remains to be determined. Certainly, PGs appear to play a significant role in normal cartilage metabolism as well as in the pathogenesis of osteoarthritis and inflammatory joint disorders (1, 2, 6). It is well recognized that during the course of osteoarthritis, proteoglycan metabolism is altered in two distinct phases. In the early stages of this disorder, aggrecan production by articular chondrocytes is markedly enhanced, presumably in an attempt to replace the matrix lost in response to physical forces and the inflammatory responses to tissue damage mediated by increased IL-1, IL-6, and tumor necrosis factor-α production (2, 6). Later in the disorder, there is apparent failure of chondrocyte function and an associated a progressive decline in aggrecan synthesis. It might be speculated that the early stimulatory phase could be associated with the local effects of E and F series PGs produced in response to inflammatory cytokine action on chondrocytes and synovial cells, resulting in the stimulation of chondrocyte aggrecan production, as observed in the present studies. In parallel, the decline in aggrecan production later in the course of the disorder might be attributable to the increasing chondrocyte dedifferentiation that occurs in the progression of osteoarthritis, perhaps related to altered extracellular matrix regulatory effects (40–42), and would result in decreased chondrocyte responsiveness to the anabolic effects of PGs. These speculations, of course, remain to be directly tested experimentally.

Our present studies extend our previous observations in the RCJ chondrocyte cell line (26, 27) and offer further indication that it is highly useful for studies of mammalian chondrocyte cellular and molecular regulation. Most previous efforts to produce chondrocytic cell lines that are capable of maintaining a differentiated phenotype in culture have relied on biochemical or oncogene transformation of primary chondrocyte isolates (43, 44). However, the use of transformed cell lines can present difficulties in extrapolating experimental results to normal tissues.

Moreover, although agarose suspension culture can induce differentiated phenotype in chondrocytes (28, 29), this system is difficult to use for routine metabolic studies due to the slow and irregular diffusion of reagents into the agarose gel and the difficulties incurred in attempting to rapidly harvest the cells and their products. In contrast, the CAG substrate culture system employed in the present studies offers rapid reagent access to cells, easy utilization of cells for metabolic studies, and the ability to induce the desired degree of cell differentiation required for a particular study. This new cell system should, therefore, prove useful for further studies of the mechanisms by which PGs and other local factors regulate chondrocyte gene expression and cellular function.

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