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Chondrocyte calcium-sensing receptor expression is up-regulated in early guinea pig knee osteoarthritis and modulates PTHrP, MMP-13, and TIMP-3 expression

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Summary

Objective: Growth plate chondrocytes up-regulate calcium-sensing receptor (CaR) expression as they mature to hypertrophy. In cells other than chondrocytes, extracellular calcium-sensing via the CaR functions partly to promote expression of parathyroid hormone-related protein (PTHrP), a critical regulator of endochondral development. Moreover, PTHrP is up-regulated in human osteoarthritis (OA) and surgically induced rabbit OA cartilages and may promote both chondrocyte proliferation and osteophyte formation therein. Hence, we examined chondrocyte CaR-mediated calcium-sensing in OA pathogenesis.

Methods: We studied spontaneous knee OA in male Hartley guinea pigs. We also evaluated cultured bovine knee chondrocytes and immortalized human articular chondrocytes (CH-8 cells), employing the CaR calcimimetic agonist NPS R-467 or altering physiologic extracellular calcium (1.8 mM).

Results: Immunohistochemistry revealed that CaR expression became up-regulated in the superficial zone at 4 months of age in the guinea pig medial tibial plateau cartilage as early OA developed. CaR expression later became up-regulated in the middle zone. PTHrP content, measured by immunoassay, was significantly increased in the medial tibial plateau cartilage as OA developed and progressed. In cultured chondrocytic cells, CaR-mediated extracellular calcium-sensing, stimulated by the calcimimetic NPS R-467, induced PTHrP and matrix metalloproteinase (MMP)-13 expression and suppressed expression of tissue inhibitor of metalloproteinase (TIMP)-3 dose-dependently, effects shared by elevated extracellular calcium (3 mM). Extracellular calcium-sensing appeared essential for PTHrP and interleukin (IL)-1 to induce MMP-13 and for PTHrP 1-34 to suppress TIMP-3 expression.

Conclusions: Chondrocyte CaR expression becomes up-regulated early in the course of spontaneous guinea pig knee OA. Chondrocyte CaR-mediated extracellular calcium-sensing promotes PTHrP expression, modulates the effects of PTHrP and IL-1, and promotes MMP-13 expression and TIMP-3 depletion. Our results implicate up-regulated extracellular calcium-sensing via the CaR as a novel mediator of OA progression.

Key words: TIMP-3, MMP-13, IL-1.

Introduction

The development of foci of chondrocyte proliferation and hypertrophy and of pathologic calcification in osteoarthritis (OA) cartilage suggests that altered differentiation of articular chondrocytes in OA at least partially recapitulates events critical to the physiologic remodeling of cartilage in the growth plate involved in mineralization[1,2]. One of the essential mediators of normal endochondral cartilage development and growth plate mineralization is locally produced parathyroid hormone-related protein (PTHrP)[3–8]. By modulating both synthesis and degradation of the matrix, as well as chondrocyte growth, differentiation, and survival, PTHrP directs the temporal and spatial organization of growth plate chondrocytes[3–6]. In this context, PTHrP regulates type II collagen, aggrecan, and link protein expression[3,5,7,8], and PTHrP also can induce matrix metalloproteinase (MMP)-2, MMP-9, and MMP-13[9,10]. PTHrP also promotes endochondral chondrocyte proliferation[3,5] and suppresses development of apoptosis[4]. PTHrP also suppresses maturation of chondrocytes to hypertrophic differentiation[3,6] via effects including modulation of expression of the runx2/cbfa1 transcription factor[11], regulation of phosphorylation and the transcriptional activity of the chondrogenic transcription factor Sox9[12], and co-ordination with Indian Hedgehog signaling[13].

We have demonstrated articular chondrocyte expression of the PTH/PTHrP receptor (also termed PTH1R) and robustly up-regulated expression of PTHrP *in situ* in human knee cartilages with advanced OA[14]. In late-stage rabbit knee OA surgically induced by partial medial meniscectomy, increased PTHrP expression developed in proliferating chondrocytes, a finding whose significance was underscored by the decrease in articular chondrocyte

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PTHrP expression observed in antigen-induced arthritis in the rabbit[15]. Recently, we observed that the human-specific PTHrP 1-173 isoform exerted potent intracrine effects on collagen synthesis and extracellular concentrations of the mineralization regulator inorganic pyrophosphate[16].

One of the major inducers of PTHrP expression identified *in vitro* in cells other than chondrocytes is the sensing of extracellular calcium by the G protein-linked calcium-sensing receptor (CaR)[17,18]. Extracellular calcium is unequivocally the primary agonist of the CaR, and, in turn, the CaR is the primary cell sensor for extracellular calcium[19,20]. In the parathyroid gland, the CaR mediates exquisitely sensitive responsiveness to extracellular calcium that exerts critical control over PTH secretion[16,18]. In the kidney, CaR expression functions as a sensing mechanism to modulate tubular ion transport[21]. But cells outside the parathyroid gland and kidney are generally less responsive to extracellular calcium than PTH-secreting cells in the parathyroid, consistent with marked effects of the environment in which the CaR sees extracellular calcium on cellular responses to calcium[19,20]. Previous studies of CaR expression and calcium-sensing in chondrogenic cell lines demonstrated suppressive effects of calcium-sensing on proteoglycans synthesis and aggrecan and type II collagen expression[22–24]. The capacity of calcium-sensing to promote chondrogenesis as well as chondrocyte maturation to hypertrophy associated with osteopontin expression and mineralization also has been demonstrated[25]. Significantly, CaR expression becomes up-regulated in association with chondrocyte hypertrophy in the growth plate[23].

Given the aforementioned observations and the fact that most normal articular chondrocytes express low levels of CaR transcripts *in situ*[23], this study examined the knee articular cartilages of Hartley guinea pigs, which spontaneously develop OA[26–28], for expression of CaR, PTHrP, and the PTH/PTHrP receptor at early and progressively more advanced time points. To better understand the potential roles played by chondrocyte PTHrP and calcium-sensing and CaR expression in OA, we investigated cultured normal bovine chondrocytes and immortalized normal human knee chondrocytic CH-8 cells[29]. We focused on the potential effects of calcium-sensing on not only PTHrP release by chondrocytic cells but also on specific chondrocyte responses that modulate OA progression [i.e., interleukin (IL)-1 responsiveness, and expression of the MMP and aggrecanase inhibitor TIMP-3 (tissue inhibitor of metalloproteinase-3)][30–32]. Our results reveal a potential direct role in the pathogenesis of OA of up-regulated extracellular calcium-sensing via enhanced chondrocyte CaR expression.

Materials and methods

REAGENTS

All chemical reagents were obtained from Sigma Chemicals (St. Louis, MO) unless otherwise specified. Synthetic PTHrP 1-34 was purchased from Bachem (Torrance, CA), and IL-1β was from R&D Systems (Minneapolis, MN). The calcimimetic CaR agonist NPS R-467[33] was generously provided by NPS Pharmaceuticals (Salt Lake City, UT). Tissue culture medium and antibiotic solutions were purchased from Mediatech (Herdon, VA).

ANIMALS

Male Hartley guinea pigs were obtained from Charles River Laboratories (Wilmington, MA) and fed with standard guinea pig chow from the same source that contained 0.85 g calcium/100 g. All animal experiments were performed in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

CARTILAGE TISSUE COLLECTION, CHONDROCYTE ISOLATION, AND CULTURE

After dissection of each hind knee in euthanized guinea pigs, uncalcified articular cartilage was removed from the tibial plateau, and samples were taken as previously described[28]. In brief, full thickness slices (<1 mm) were cut from the medial central hyaline cartilage zone not covered by menisci (width × length; 2 × 3 mm) and the meniscal fibrocartilage zone (3 × 3 mm) of 2-, 4-, and 8-month-old animals. Washed cartilage samples were cultured in Dulbecco's modified Eagle's (DME's) medium (high glucose), supplemented with 2 mM L-glutamine, 100 Units/ml penicillin, and 50 μg/ml streptomycin ("standard growth medium"), and heat-inactivated 1% fetal bovine serum (FBS). Where indicated, guinea pig knee chondrocytes were isolated and cultured in the same medium, as described in detail[28], specifically for assays of PTHrP release. The cartilage slices were extracted in 50 mM Tris, pH 7.5, 0.25 M NaCl, 0.1% NP 40, 1% Triton X-100, 5 mM ethylenediaaminetetraacetic acid (EDTA), and 5 mM NaF, and were stored at −70°C until further processing.

Bovine chondrocytes were isolated by collagenase digestion of mature normal knees (Animal Technologies, Tyler, TX), and cells were plated in standard growth medium for 7 days before evaluating the cells in functional studies in monolayer, as described[34].

Clonal human chondrocytic cells (CH-8) from adenoviral SV40 early gene-immortalized normal knee articular chondrocytes[29] exhibited continuous proliferative capacity in monolayer culture and maintained chondrocytic differentiation, verified by expression of collagen type II and aggrecan detected by reverse transcription-polymerase chain reaction (RT-PCR). Here, CH-8 cells (passages 4−10) were studied in monolayer culture in dishes coated with type II collagen in standard growth medium, supplemented with 10% FBS.

IMMUNOHISTOCHEMISTRY

Guinea pig knees were dissected, fixed in 10% formaldehyde/HCl to decalcify the bone and paraffin-embedded 6 μm sections from the central portion of the medial tibial plateau articular cartilage were microtomed and placed onto (+) charged microscope slides (Fisher Scientific, Pittsburgh, PA), as previously described[28]. Sections were deparaffinized in xylene and hydrated through a graded ethanol series. Safranin O staining was performed on the sections to identify the cartilage. Sections were heated at 95°C in 10 mM sodium citrate buffer, pH 6.0, for antigen retrieval and quenching of endogenous horseradish peroxidase activity. Nonspecific protein binding was blocked with 20% FBS, 0.25% gelatin in phosphate buffered saline (PBS) for 30 min. Sections were incubated with primary antibodies for PTHrP (the murine PTHrP antibody 9H7)[14], the PTH/PTHrP receptor (generously provided by Dr. Mitch Scott, Washington Univ., St. Louis, MO), and CaR (Affinity Bioreagents, Golden, CO) diluted in 0.25% gelatin in PBS overnight at 4°C. Biotinylated goat anti-mouse or rabbit antibody (Calbiochem, San Diego, CA), diluted at 1:200

with 0.25% gelatin in PBS, was applied for 2 h. The slides were washed and then incubated with streptavidin–horseradish peroxidase conjugate. The staining was developed with the chromogen 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide in 25 mM Tris, pH 7.4. Negative controls included omitting the primary antibody and, for PTHrP, preadsorbing the primary antibodies with 100 molar excess of immunizing antigen. Positive controls for immunostaining included sections from paraffin-embedded human lung cancer-derived BEN cells and sections from paraffin-embedded osteogenic sarcoma-derived SaOS-2 cells.

PTHrP IMMUNOASSAY

Guinea pig cartilage explants and bovine chondrocyte and CH-8 cell conditioned media were measured for PTHrP by radioimmunoassay[14,16]. In brief, human PTHrP 1-34 peptide was used as standard and PTHrP 1-86 peptide was radioiodinated by the chloramine T method. A rabbit PTHrP antibody (R869) directed against PTHrP 1-34 was used for the measurements of PTHrP levels in 3 days non-equilibrium immunoassay format, as described[14]. All samples were assayed in triplicate in multiple dilutions that paralleled the corresponding PTHrP standard curve. The intra- and inter-assay variations were approximately 7–12%, and the limit of assay sensitivity was 4 pmol per liter. The unknown sample B/Bo values were on the linear portion of the radioimmunoassay standard curve using 100 μl sample/tube. Lack of cross-reactivity in the assay for at least a 100-fold excess of peptide was demonstrated for non-corresponding PTHrP peptides, PTH 1-84, calcitonin, calcitonin gene-related peptide, and human and rat atrial natriuretic peptide.

STUDIES OF CALCIUM-SENSING IN CULTURED CELLS

Aliquots of 3.0×10^5 CH-8 cells and bovine chondrocytes in 6-well culture plates pre-coated with type II collagen were placed in DME formulated with no calcium salts (DME-no Ca) (Invitrogen, Carlsbad, CA). IL-1β (10 ng/ml) and PTHrP 1-34 (10 nM) were added to the DME-no Ca^{2+} medium supplemented with $CaCl_2$ (0.5 mM, 1.8 mM, or 3.0 mM) and 1% FBS, 250 Units penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. For NPS R-467 studies, cells were treated in DME-no Ca^{2+} supplemented with 1% FBS and 0.5 mM Ca^{2+}, the media replaced at 3 days in culture, and at 5 days, conditioned media were collected and cell lysates were collected in 150 mM NaCl, 0.5 mM EDTA, 1 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1% sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate, and supplemental protease inhibitors in 10 mM Tris HCl, pH 7.6.

WESTERN BLOT ANALYSES

Aliquots containing 15 μg of protein/well were separated by 10% SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), as described[34]. Antibodies to MMP-13 (Oncogene, San Diego, CA) and TIMP-3 (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:100 and 1:1000, respectively, in blocking buffer and incubated for 18 h at 4°C. Secondary antibodies [goat anti-rabbit or anti-mouse conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA)] were at 1:1000. Optical densities of bands were quantified using the FluorChem 8900 image analysis system (Alpha Innotech, San Leandro, CA).

RT-PCR

Expression of PTHrP, PTH/PTHrP receptor and CaR messenger RNAs (mRNAs) were evaluated by RT-PCR analyses on total RNA, as described[14]. All PCR products were sequenced to confirm the fidelity of amplification. Primer sets used were: CaR: forward 5′-GCAACACAC CCATTGTCAAG-3′ and reverse 5′-GGCAAAGAAGAA GCAGATGG-3′, PTH/PTHrP receptor: forward 5′-GTG AACGGGAGGTGTTTGA-3′ and reverse 5′-CCCGGACGA TATTGATGAAG-3, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH): forward 5′-GGAGTCAACGGATT TGGT-3′ and reverse 5′-GTGATGGGATTTCCATTG AT-3′.

STATISTICS

Statistical analyses were performed using Microsoft Excel (Microsoft Corp., Redmond, WA) and Statview (SAS, Cary, NC) software, with results reported as mean ± S.E.M. and differences among treatment groups assessed using single factor analysis of variance.

Results

PTHrP, PTH/PTHrP RECEPTOR, AND CAR EXPRESSION IN GUINEA PIG KNEE CARTILAGE

Spontaneous knee OA in male Hartley guinea pigs appears first in the medial tibial plateau articular cartilage[26–28]. The earliest changes in cartilage, including surface lesions, typically are seen at 3–4 months of age, with moderate OA developing by 6–12 months of age in the medial tibial plateau cartilage[26–28], findings that we confirmed here. In brief, we examined sections from 2-, 4- and 8-month-old Hartley guinea pig knee medial tibial plateau articular cartilages. At 2 months of age, we confirmed that the medial tibial plateau articular cartilage was grossly normal with a smooth surface (not shown), and histologically, the same sites of cartilages of 2-month-old animals also had a normal appearance, as assessed by Safranin O staining (Fig. 1). By 4 months of age, minor cartilage surface irregularity was confirmed[28] on gross examination (not shown), and histologic changes confirming OA in the medial tibial plateau cartilage included decreased Safranin O staining at the cartilage surface, the development of vertical columns of chondrocytes (palisades), and moderately decreased cellularity in the superficial zone (Fig. 1). At 8 months of age, gross surface erosions were detectable in medial tibial plateau cartilage (not shown), and histologic changes included marked decreases in both cellularity and Safranin O staining in the superficial zone, as well as palisading of chondrocytes in the middle zone (Fig. 1). We did not histologically examine the knee menisci in this study.

Immunohistochemistry for PTHrP, PTH/PTHrP receptor, and CaR in serial sections from the same 2-, 4- and 8-month-old guinea pig knees revealed that PTHrP expression was markedly up-regulated in the superficial zone in cartilages at 8 months of age, whereas CaR expression was markedly up-regulated in the superficial zone at 4 months of age, and the up-regulation of CaR expression extended to the middle zone at 8 months of age (Fig. 1). In

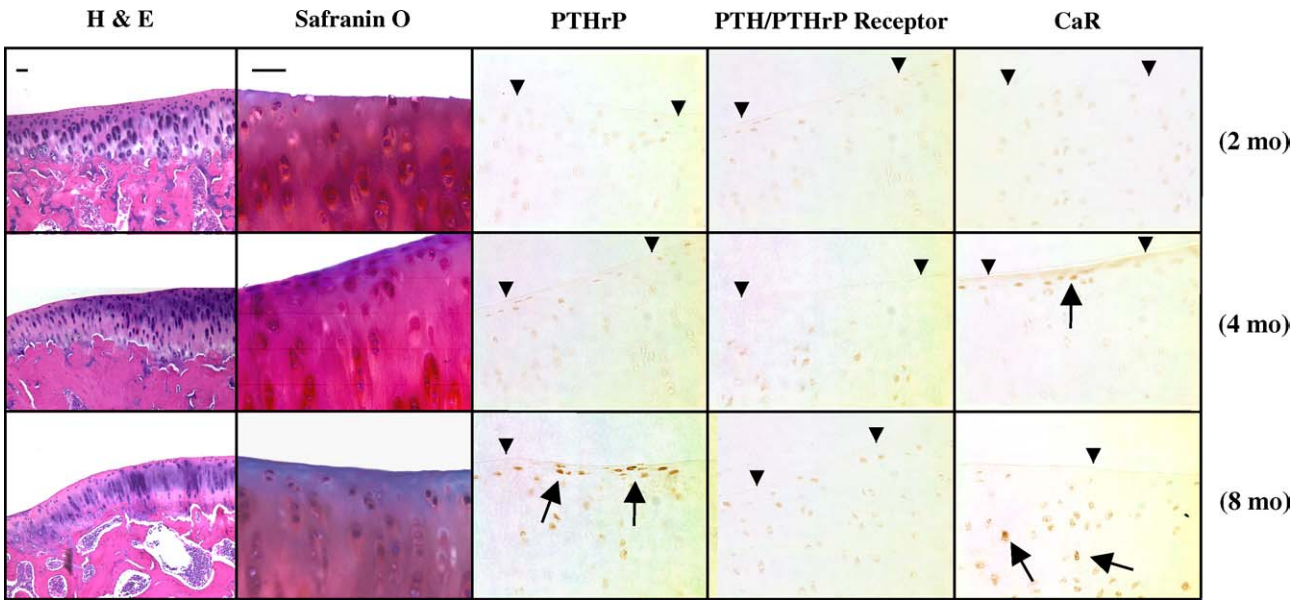

Fig. 1. Up-regulation of PTHrP and CaR expression as OA evolves in medial tibial plateau articular cartilage in the Hartley guinea pig knee. Tibiae from 2-, 4- and 8-month-old guinea pigs were dissected, decalcified and embedded into paraffin, and medial tibial plateau central zone articular cartilage sections (6 μm) were stained with hematoxylin/eosin (H & E) and Safranin O (left side panels) or analyzed by immunohistochemistry for PTHrP, PTH/PTHrP receptor and CaR, as described in the Methods. Cartilages of 2-month-old animals had a normal histologic appearance on H & E and Safranin O staining, but by 4 months of age, histologic changes confirming OA in the medial tibial plateau cartilage included decreased Safranin O staining at the cartilage surface, the development of vertical columns of chondrocytes (palisades), and moderately decreased cellularity in the superficial zone. At 8 months of age, histologic changes included a marked decrease in both cellularity and Safranin O staining in the superficial zone, as well as palisading of chondrocytes in the middle zone. The increased PTHrP and CaR expression were detected as brown precipitates in the medial tibial plateau chondrocytes at 4 and/or 8 months of age, as indicated (arrows). Negative controls (not shown) included omitting the primary antibody and, for PTHrP, preadsorbing the primary antibodies with 100 molar excess of immunizing antigen. Positive controls for immunostaining (not shown) included sections from paraffin-embedded human lung cancer-derived BEN cells and sections from paraffin-embedded osteogenic sarcoma-derived SaOS-2 cells. No significant change was observed for the PTH/PTHrP receptor immunostaining in the chondrocytes over the evolution of OA and the negative controls did not demonstrate any specific staining in the chondrocytes (data not shown). The arrowheads in the immunohistochemistry panels indicate the cartilage surface. Top left panel bar = 50 μm. Staining experiments were performed on guinea pig sections from at least three animals per time point and staining was repeated at least once for each animal.

contrast, PTH/PTHrP receptor expression in the superficial and middle zones did not appreciably change over the same time period.

Next, we quantitatively analyzed PTHrP expression by immunoassay in the tissue extracts of dissected medial tibial plateau central zone hyaline cartilage and of meniscal fibrocartilage from the knees of 2-, 4- and 8-month-old animals. Relative to normal samples at 2 months of age, total PTHrP content was significantly increased in whole tissue extracts from the medial tibial plateau hyaline cartilage at 4 months of age and 8 months of age (though less abundant than at 4 months of age) (Fig. 2). Age-related differences in PTHrP release in the conditioned media from cultured chondrocytes isolated from guinea pig knee articular hyaline cartilages were comparable to those seen with the whole cartilage extracts (not shown). Meniscal fibrocartilage extracts also demonstrated a significant increase in PTHrP content, but this appeared only in samples from 8-month-old animals (Fig. 2). Hence, chondrocyte PTHrP expression changed as OA evolved over time in the Hartley guinea pig knee.

FUNCTIONAL IMPLICATIONS OF CALCIUM-SENSING IN CHONDROCYTIC CELLS

To better understand how calcium-sensing by chondrocytes might modulate OA, we initially studied first passage normal bovine articular chondrocytes and also employed clonal, SV40 transformed chondrocytes from normal human knee cartilage (CH-8 cells)[29]. Using RT-PCR, we observed that the CH-8 cells (Fig. 3) and normal bovine knee chondrocytes (not shown) expressed PTHrP, PTH/PTHrP receptor, and CaR. Next, we incubated the chondrocytes in medium containing subphysiologic, physiologic, and supraphysiologic extracellular calcium concentrations (0.5, 1.8, 3.0 mM Ca^{2+}, respectively). In parallel studies, we employed NPS R-467 (0–10 nM in medium containing 0.5 mM Ca^{2+}). NPS R-467 is a phenylalkylamine that acts as a CaR agonist via allosterically modifying CaR function by binding to CaR transmembrane domains and increasing apparent affinity of the CaR for Ca^{2+} (Ref.[33]).

PTHrP secretion was increased at physiologic and supraphysiologic extracellular Ca^{2+} concentrations relative to the subphysiologic concentration of 0.5 mM Ca^{2+} in bovine chondrocytes [Fig. 4(A)]. Furthermore, treatment with the calcimimetic NPS R-467 directly stimulated PTHrP secretion in bovine chondrocytes [Fig. 4(B)]. Specifically, there was a ~3-fold increase in PTHrP release at 1 nM NPS R-467 and a ~15-fold increase at 10 nM NPS R-467 compared to vehicle treated control. Because IL-1 was previously established to stimulate PTHrP secretion by human chondrocytes[14], we next assessed for potential effects of calcium-sensing on IL-1β-induced PTHrP secretion in bovine chondrocytes. Treatment with IL-1 increased

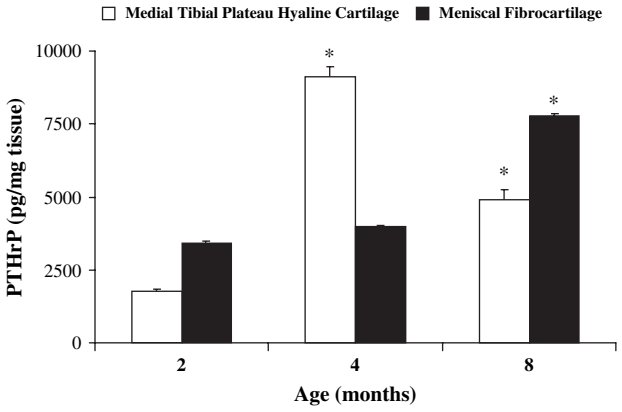

Fig. 2. Changes in cartilage PTHrP content as OA evolves in the Hartley guinea pig. Freshly dissected cartilage sections from 2-, 4-, and 8-month-old Hartley guinea pigs were weighed and incubated *in situ* for 48 h at 37°C in growth medium supplemented with 1% serum, and PTHrP levels were measured in whole tissue extracts by immunoassay, as described in the Methods. We sampled the medial tibial plateau central zone of hyaline cartilage uncovered by meniscus, as this zone develops the earliest OA lesions. We concurrently sampled medial and lateral meniscal fibrocartilage from the same knees. The asterisks (*) indicate significant differences ($P < 0.05$) compared to the 2-month-old animals. $n = 12$ animals for each type of specimen at each age.

the secretion of PTHrP by chondrocytes and this activity was potentiated by increasing concentrations of extracellular Ca^{2+} or of NPS R-467 [Fig. 4(A–B)].

Treatment with supraphysiologic extracellular calcium concentration or with calcimimetic also inhibited TIMP-3 expression in bovine chondrocytes (Fig. 5). Specifically, TIMP-3 expression was inhibited by ~50% in medium

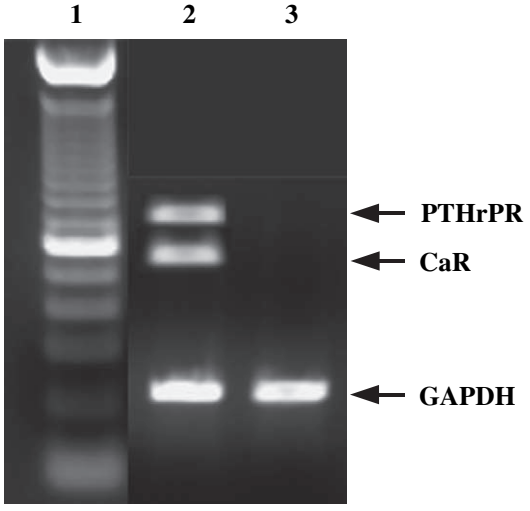

Fig. 3. Chondrocytic CH-8 cells express PTH/PTHrP receptor mRNA and CaR mRNA. RT-PCR for PTH/PTHrPR (parathyroid hormone-related protein receptor), CaR and GAPDH was performed on the Ch-8 cells (lane 2) as described in the Methods. The PCR products generated from the cell lines using primers specific for the PTHrP receptor, CaR and GAPDH were consistent with the predicted product sizes, 615 bp, 461 bp and 206 bp, respectively. A 100 bp DNA molecular weight ladder is shown on the left (lane 1). Lane 3 had no PTH/PTHrPR or CaR primers added to the PCR reaction.

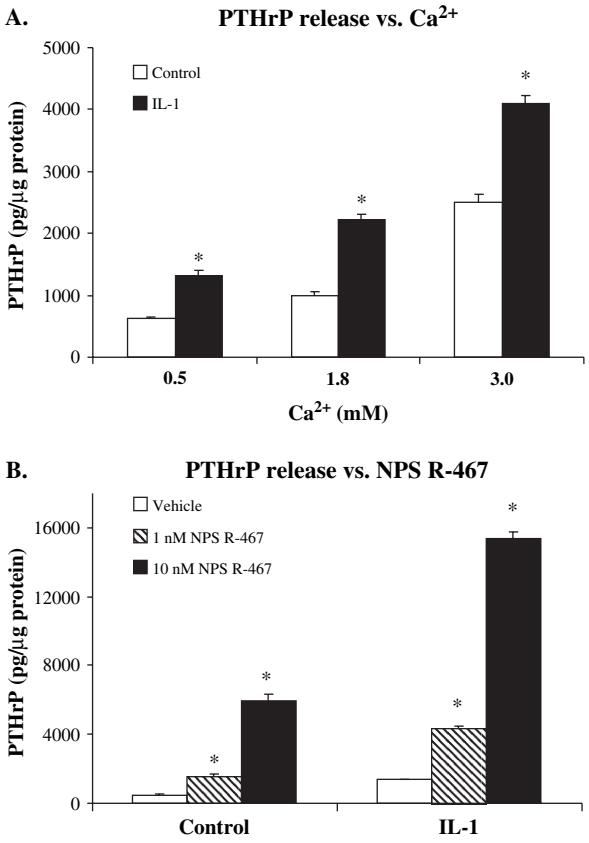

Fig. 4. PTHrP release in response to differing concentrations of extracellular calcium or the CaR agonist (calcimimetic) NPS R-467 in cultured bovine articular chondrocytes. Aliquots of 3.0×10^5 first passage normal bovine chondrocytes were cultured as described in the Methods and treated as indicated with subphysiologic, physiologic (1.8 mM), and supraphysiologic concentrations of extracellular calcium or of the CaR agonist (calcimimetic) NPS R-467 in 0.5 mM extracellular calcium. On the fifth day of culture, the conditioned media were collected and PTHrP 1-34 levels measured by immunoassay. Data are pooled from of eight separate experiments performed in duplicate. For panel A, asterisks (*) indicate significant differences ($P < 0.05$) in PTHrP release as compared to no stimulation at individual calcium concentrations. There also was a significant difference ($P < 0.05$) in PTHrP release among control groups at calcium concentrations other than physiologic (1.8 mM). For panel B, asterisks (*) indicate significant differences ($P < 0.05$) in PTHrP release as compared to buffer alone with no NPS R-467.

containing 3.0 mM Ca^{2+} compared to medium containing 0.5 mM Ca^{2+}, but was induced relative to vehicle control alone by PTHrP treatment in cells in 3.0 mM Ca^{2+} [Fig. 5(A, B)]. Treatment of the cells with NPS R-467 dose-dependently inhibited TIMP-3 expression under all conditions, and was associated with failure of PTHrP to induce TIMP-3 [Fig. 5(C, D)]. For example, 10 nM NPS R-467 inhibited TIMP-3 expression by >80% compared to vehicle treated control cells [Fig. 5(C, D)].

To further understand the effects of calcium-sensing on modulation of matrix catabolism, we concurrently assessed MMP-13 and TIMP-3 expression. Because of limitations related to cross-reactivity of available antibodies with bovine MMP-13 (Foster, M, *et al.*, unpublished observations), we carried out these studies in the human clonal CH-8 chondrocytic cells (Fig. 6). We observed that supraphysiologic extracellular Ca^{2+} stimulated release of

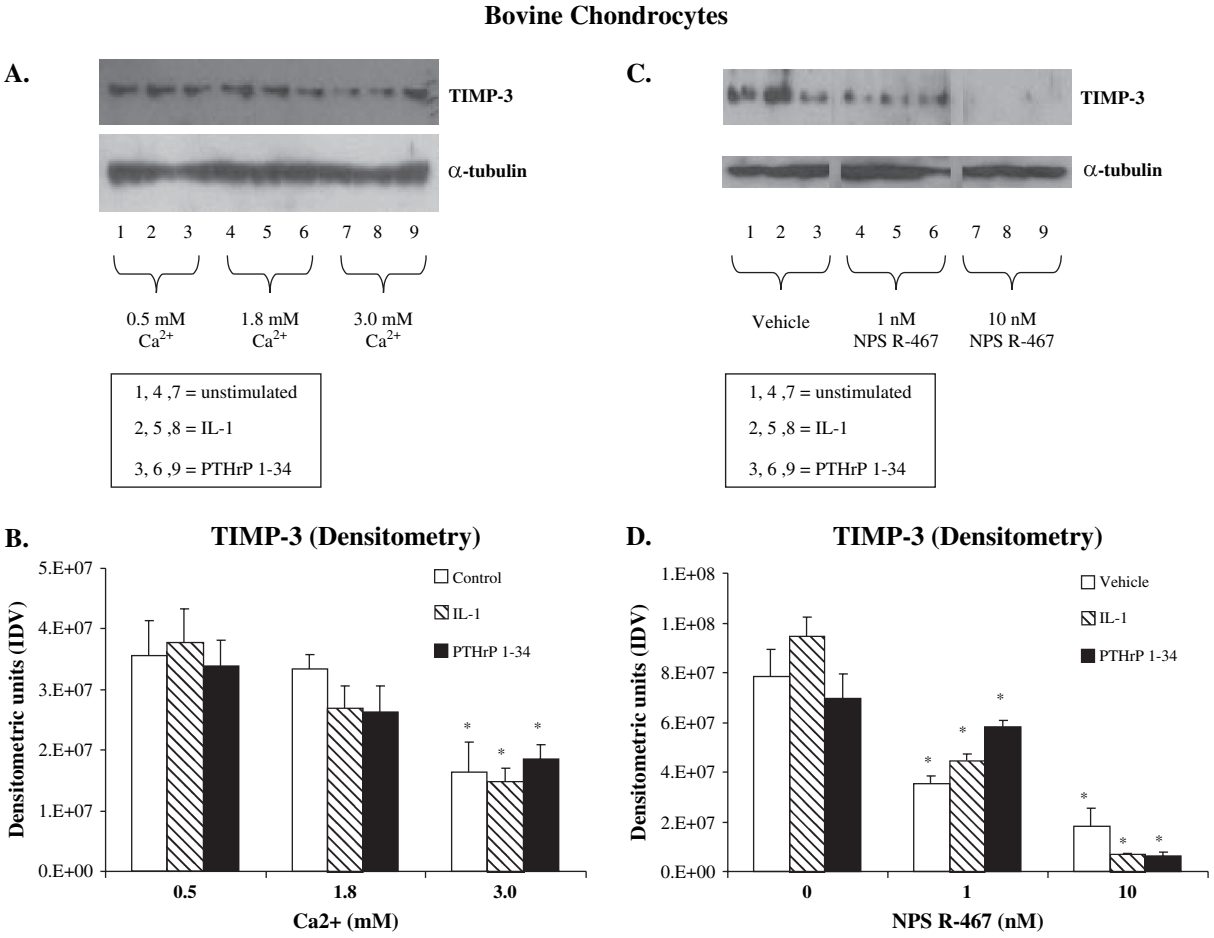

Fig. 5. TIMP-3 expression in response to differing concentrations of extracellular calcium and NPS R-467 in bovine chondrocytes. Aliquots of 3.0×10^5 first passage bovine chondrocytes were treated as described above and cell lysates collected on the fifth day of culture were analyzed by SDS-PAGE/Western blotting for TIMP-3 and alpha-tubulin and quantified by densitometry as described in the Methods. Results are expressed as the integrated densitometric value (IDV) for the densitometric units of TIMP-3 normalized to alpha-tubulin. The asterisks (*) indicate significant differences ($P < 0.05$) for TIMP-3 relative to the control (physiologic calcium concentration (1.8 mM) open bar, panel B) or vehicle only (no NPS R-467 added, open bar, panel D). Densitometry results shown are representative of five separate experiments and the representative Western image for panel C was spliced together from the same gel.

MMP-13 pro-enzyme, and the lower molecular weight form of MMP-13 (activation band) also appeared in the conditioned medium of cells treated with supraphysiologic extracellular Ca^{2+} [Fig. 6(A)]. The calcimimetic stimulated release of MMP-13 pro-enzyme, but did not stimulate formation of the MMP-13 activation band [Fig. 6(D)]. Supraphysiologic extracellular Ca^{2+} inhibited TIMP-3 expression, but PTHrP demonstrated induction of TIMP-3 in the presence of supraphysiologic extracellular Ca^{2+} [Fig. 6(A)]. Treatment with the calcimimetic suppressed TIMP-3 expression under all conditions studied [Fig. 6(A, C, D, F)]. Neither IL-1 nor PTHrP was able to induce MMP-13 at the subphysiologic extracellular Ca^{2+} concentration of 0.5 mM, but either increasing extracellular Ca^{2+} or adding the calcimimetic allowed both IL-1 and PTHrP to induce MMP-13 [Fig. 6(A, B, D, E)].

Discussion

In this study, we demonstrated marked up-regulation in the expression of both the CaR and PTHrP in the course of spontaneous knee OA in the Hartley guinea pig knee joint. In the superficial zone of the medial plateau of the Hartley guinea pig knee, the site of the earliest OA lesions in this model[26], up-regulation of CaR expression preceded that of PTHrP expression. In human cartilage with advanced OA, PTHrP is up-regulated[14]. Here, we demonstrated PTHrP to be sequentially up-regulated in the superficial zone and then the middle zone of the Hartley guinea pig knee medial plateau cartilage. PTHrP is a critical regulator of growth plate development[3,6]. Hence, PTHrP may be one of the factors driving the chondrocyte proliferation ("chondrocyte cloning") known to develop in OA cartilage[35], and up-regulated PTHrP co-localized with proliferating chondrocytes in established, surgically induced OA in rabbit knees[15]. PTHrP also might help ignite the endochondral developmental cascade involved in osteophyte formation[36]. But by promoting chondrocyte MMP-13 expression[10], as reinforced in this study, PTHrP also can contribute to cartilage matrix degradation in OA[37].

This study demonstrated CaR expression by first passage normal chondrocytes from bovine cartilage, as well as chondrocytic human CH-8 cells, which permitted

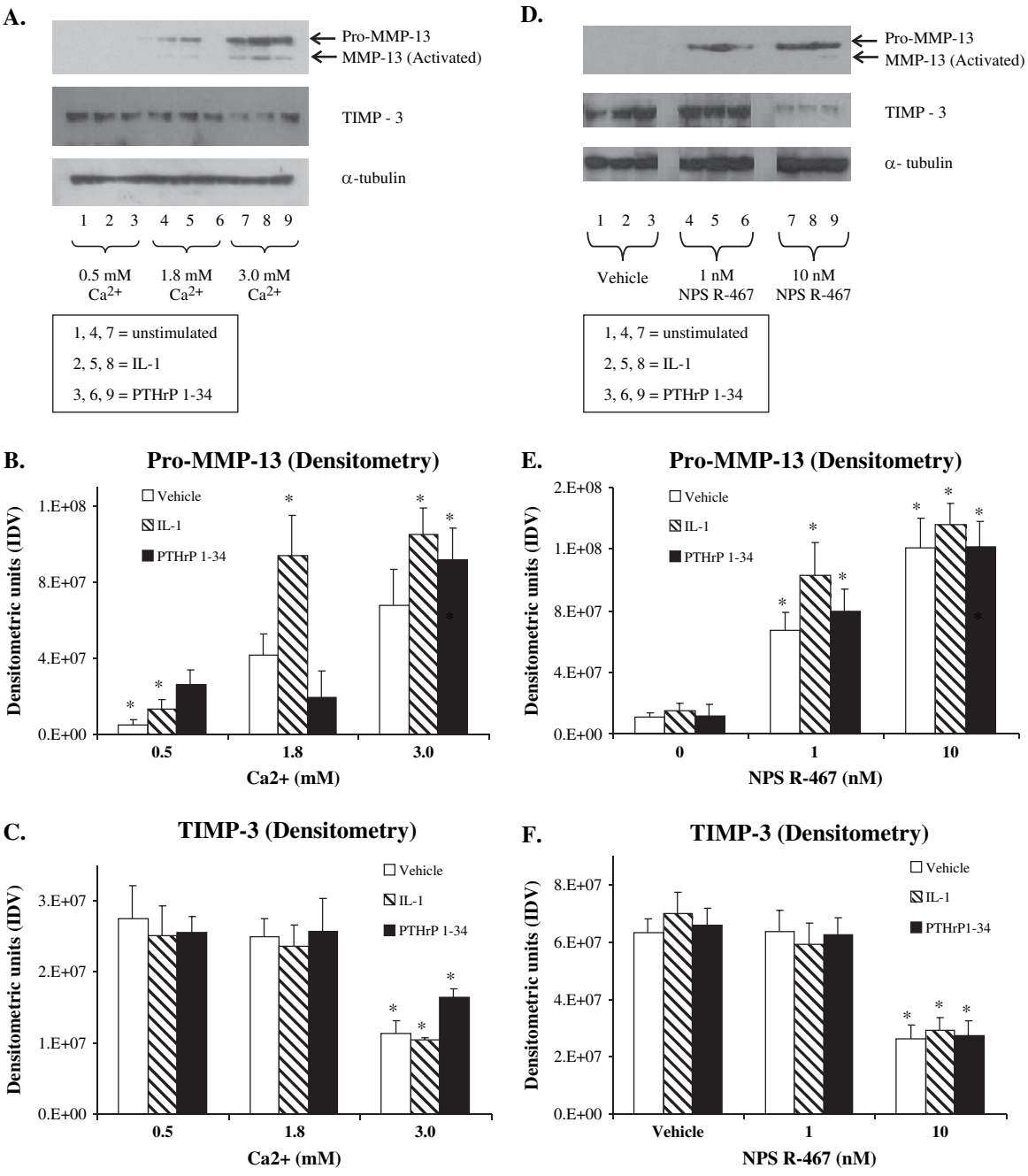

Fig. 6. MMP-13 and TIMP-3 expression in response to higher concentrations of calcium in CH-8 cells. Aliquots of 3.0×10^5 CH-8 cells were cultured as described in the Methods and on the fifth day of culture, the conditioned media were collected for SDS-PAGE/Western blot analyses of MMP-13 and cell lysates were collected for SDS-PAGE/Western blot analyses of TIMP-3 and alpha-tubulin (panels A and D). The Western blots were quantified by densitometry and results are expressed as densitometric units of TIMP-3 normalized to alpha-tubulin, as above, or non-normalized densitometric units of MMP-13 pro-enzyme and the MMP-13 activation band. For panels B and C, the asterisks (*) indicate significant differences ($P < 0.05$) in expression of pro-MMP-13 (panel B) or of the TIMP-3 band (panel C) as compared to control (physiologic calcium (1.8 mM) open bar). For panels E and F, the asterisks (*) indicate significant differences ($P < 0.05$) in expression of pro-MMP-13 (panel E), and of TIMP-3 (panel F) as compared to the respective vehicle control (no NPS R-467 added, open bar). Densitometry results shown are pooled from five separate experiments.

investigation of functional aspects of calcium-sensing in chondrocytic cells pertinent to pathogenesis of OA. A noteworthy aspect of the experimental design was the parallel study of altered extracellular calcium and of the calcimimetic NPS R-467. We demonstrated that one of the functions of calcium-sensing via the CaR in chondrocytes was to stimulate PTHrP expression. We also observed that calcium-sensing via the CaR directly promoted MMP-13 expression and suppressed expression of the MMP and aggrecanase inhibitor TIMP-3. The significance of the observed suppressive effects of CaR-mediated calcium-sensing on expression of TIMP-3 was underscored

by the lack of detectable TIMP-1 or TIMP-2 expression in the cells cultured in this study (Foster, M, unpublished observations). In the presence of supraphysiologic extracellular calcium but not the calcimimetic, both MMP-13 proenzyme and the MMP-13 activation band were induced. These results suggested effects of extracellular calcium on MMP-13 beyond cellular calcium-sensing, which may have been exerted at the level of MMP-13 conformation and MMP-13 susceptibility to post-translational modifications including proteolysis[38]. Last, we observed that the CaR calcimimetic potentiated the capacity of both PTHrP and IL-1 to promote MMP-13 expression and suppress TIMP-3 expression. Interestingly, TIMP-3 expression was not suppressed in response to PTHrP in bovine chondrocytes and CH-8 cells cultured in supraphysiologic extracellular calcium, which was not the case in the cells treated with the calcimimetic. We speculate that PTHrP effects on TIMP-3 expression might be subject to influence by cytosolic calcium currents in addition to extracellular calcium-sensing.

Though the role of IL-1 in the induction of OA is not entirely resolved, IL-1 appears to play a substantial role in promoting cartilage matrix degradation in established OA[39,40]. The induction of p44/42 mitogen-activated protein kinase signaling and c-fos expression are among the signals that transduce responses including expression of certain MMPs in IL-1-treated chondrocytes[41,42]. In this context, IL-1 signaling has previously been observed to require calcium uptake to activate p44/42 signaling and c-fos expression, and extracellular calcium depletion decreases these particular responses[42]. Cellular uptake of calcium, as opposed to membrane calcium-sensing, has been reported to modulate the expression of certain MMPs[43,44]. But it is noteworthy that one of the responses mediated by calcium-sensing via the CaR is modulation of cellular calcium uptake[45]. It also is conceivable that extracellular calcium concentrations might rise in some micro-environments within articular cartilage in which calcific crystalline deposits of hydroxyapatite and calcium pyrophosphate dihydrate are formed or resorbed, and such pathologic calcification is a common occurrence in degenerative joint disease[46].

The results of this study reinforce the importance of both extracellular calcium concentration and CaR-mediated calcium-sensing for maximal induction of MMP-13 expression by IL-1, and additionally by PTHrP. It will be of interest to determine if CaR-mediated calcium-sensing modulates protein kinase A pathway activation, which has previously been observed to transduce the capacity of PTHrP 1-34 to stimulate MMP-13 expression in chondrocytes[47].

The primary agonist of the CaR is extracellular calcium, and, conversely, the CaR acts as the principal cell sensor for extracellular calcium[19,20]. However, the effective concentration to cause 50% of the mean maximum response (EC_{50}) for calcium to activate the CaR is typically supraphysiologic (3–4 mM) in cells cultured from most types of tissues[19,20], In this regard, alternative, higher affinity ligands to calcium for the CaR also exist, including the polyamine spermine, and the divalent cations Zn^{2+} and Fe^{2+} (Refs.[19,20]). As such, there are open questions about the breadth of potential housekeeping functions and degenerative joint disease-related functions of the CaR and the range of CaR ligands involved, particularly given the demonstration herein of up-regulated CaR expression in spontaneous knee OA in the Hartley guinea pig.

The focused framework of the current study did not extend to examination of chondrocyte calcium influx dependent or independent of the CaR, or to G protein-receptor-coupled signal transduction potentially mediated by CaR[17–20] within chondrocytic cells, and we did not evaluate MMP-13, TIMP-3, or PTHrP transcriptional regulation. In addition, we have not examined the effects of calcium-sensing on MMP-13 post-translational processing or net MMP-13 catalytic activity. This leaves open for future study several compelling questions, given the observed concurrent effects on MMP-13 and TIMP-3 released by chondrocytic cells and the larger significance of MMP-13 processing and of TIMP-3 regulatory effects on MMP-13 activity[48–51] and cartilage metabolism[52]. Nevertheless, this study has provided novel findings of dysregulated CaR expression in early guinea pig knee OA, of dysregulated PTHrP expression in the same setting, and of significant effects of calcium-sensing via the CaR on PTHrP expression and on the regulation of MMP-13 and TIMP-3 expression. Our results indicate the potential of dysregulated CaR in early OA to contribute to the pathogenesis and progression of the disease.

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