

Differential Effects of Parathyroid Hormone Fragments on Collagen Gene Expression in Chondrocytes

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Abstract. The effect of parathyroid hormone (PTH) in vivo after secretion by the parathyroid gland is mediated by bioactive fragments of the molecule. To elucidate their possible role in the regulation of cartilage matrix metabolism, the influence of the amino-terminal (NH₂-terminal), the central, and the carboxyl-terminal (COOH-terminal) portion of the PTH on collagen gene expression was studied in a serum free cell culture system of fetal bovine and human chondrocytes. Expression of $\alpha 1$ (I), $\alpha 1$ (II), $\alpha 1$ (III), and $\alpha 1$ (X) mRNA was investigated by in situ hybridization and quantified by Northern blot analysis. NH₂-terminal and mid-regional fragments containing a core sequence between amino acid residues 28-34 of PTH induced a significant rise in

$\alpha 1$ (II) mRNA in proliferating chondrocytes. In addition, the COOH-terminal portion (aa 52-84) of the PTH molecule was shown to exert a stimulatory effect on $\alpha 1$ (II) and $\alpha 1$ (X) mRNA expression in chondrocytes from the hypertrophic zone of bovine epiphyseal cartilage. PTH peptides harboring either the functional domain in the central or COOH-terminal region of PTH can induce cAMP independent Ca²⁺ signaling in different subsets of chondrocytes as assessed by microfluorometry of Fura-2/AM loaded cells. These results support the hypothesis that different hormonal effects of PTH on cartilage matrix metabolism are exerted by distinct effector domains and depend on the differentiation stage of the target cell.

PARATHYROID hormone plays a predominant role in the regulation of calcium homeostasis by acting mainly on its target tissues in the renal cortex and bone (15, 42). Soon after secretion the parathyroid hormone (PTH)¹ molecule undergoes rapid proteolysis in the liver resulting in multiple fragments (7). Since most of the calcium regulatory functions could be mapped to the NH₂-terminal portion (PTH 1-34) of PTH, it was thought that this fragment contains all structural requirements for biological activity of the entire molecule (43, 52). The other fragments were regarded as inactive metabolites whose functional importance was confined to processing and intracellular transport events during hormone secretion by the cells of the parathyroid gland (PTH 53-84, references 35, 46). However, there is now increasing evidence for a broader spectrum of target tissues, including cartilage (26, 33, 34), and of hormone action in growth (30, 48) and dif-

ferentiation processes (8, 13) which are mediated by additional functional domains on the mid-regional (23) and COOH-terminal portion (39, 40, 44) of PTH. For example, PTH (53-84) increases alkaline phosphatase activity in osteoblastic cell lines (39) and more recent studies showed that PTH (39-84) and PTH (53-84) dose dependently stimulate the differentiation of osteoclast precursors into osteoclast-like cells (25). Moreover, for two domains of PTH their functional role in the induction of second messenger pathways has been elucidated: the first two NH₂-terminal amino acids of PTH are needed for adenylate cyclase stimulation via the "classical" PTH receptor (24, 43, 52), whereas the mid-regional part, aa 28-34, is responsible for induction of protein kinase C activation in target cells (23). This domain in the central part of PTH also seems to be critical for the mitogenic effect of the fragments PTH (28-48) and (1-34) in primary cultures of sternal embryonic chicken chondrocytes (48). A more complex pattern of PTH effects has been demonstrated in a culture system of neonatal murine mandibular condylar explants exposed simultaneously to PTH fragments 1-34, 28-48, and 53-84 (51). Each of these fragments was shown to exert distinct biological effects (51) on the cartilage

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1. *Abbreviations used in this paper:* PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide.

morphology, indicating a potential critical role for PTH in normal endochondral ossification.

In contrast to these well characterized PTH effects on cell numbers, cell shape and extracellular matrix morphology, little is known about the ability of the different PTH fragments to induce quantitative and/or qualitative changes in collagen gene expression by chondrocytes. Therefore, it was the aim of this study to elucidate the regulatory potential of different PTH fragments on collagen metabolism of chondrocytes, which might be a critical underlying mechanism of PTH action on cartilage.

Several studies (8, 13, 30) have indicated that the response of chondrocytes to PTH depends on the source and developmental stage of the cartilage. In embryonic transient cartilage, such as epiphyseal cartilage of long bone rudiments, chondrocytes rapidly proliferate and undergo a series of differentiation steps. These stages of chondrocyte differentiation are aligned sequentially from the epiphyseal surface down to the growth zone in the diaphysis and are characterized by the expression of different collagen types as specific markers (for review see reference 55). Preembryonic cells in the superficial layer express collagen type I, proliferating chondrocytes in the middle zone synthesize collagen II, VI, IX, and XI, whereas hypertrophic chondrocytes of the growth plate can be unequivocally identified on the basis of their collagen type X expression (16, 49). The expression of this characteristic collagen in the deep zone of the epiphysis seems to be functionally related to endochondral ossification processes (16, 49) in the matrix preceding cartilage resorption by osteoclasts and replacement by endochondral bone. Since PTH is known to promote endochondral ossification (50), it was the aim of our study to investigate modulatory effects of PTH fragments on the expression of collagen II and X mRNA in epiphyseal chondrocytes of different developmental stages.

Materials and Methods

Chemicals and Supplies

Bovine (b) and human (h) PTH fragments were obtained from Sigma (St. Louis, MO): h, b(PTH) bPTH (1-34), (Nle^{8,18}, Tyr³⁴) bPTH (3-34), hPTH (13-34), hPTH (28-48), hPTH (39-68), (Tyr⁵², Asn⁷⁶) hPTH (52-84), and hPTH (64-84). Tissue culture supplies were purchased from Becton Dickinson (NJ), and FCS from PAA-Labor, Forschungs GmbH (Linz, Austria).

Agarose Cell Culture

Juvenile human costal cartilage obtained from funnel chest operations was dissected free of surrounding tissues and cut into 0.5-mm slices. Chondrocytes were released by collagenase digestion and cultured in agarose gels under serum-free conditions as described previously (6, 53). Briefly, matrix-free cells suspended in media containing 0.5% of low melting agarose were seeded into prewarmed culture dishes coated with 1% high melting agarose gels. The cultures were maintained at 37°C to keep the low melting agarose in the liquid state and, thus, to allow the cells to sediment at the interface of the two agarose layers. Thereafter, the low melting agarose was allowed to gel by brief exposure of the cultures to 4°C. Cultures were then supplemented with additional medium. Cells were grown at densities of 2×10^6 /ml in DMEM (Grand Island Biologicals Corp., Basel, Switzerland) containing 60 µg/ml of β-aminopropionitrile fumarate, 50 µg/ml sodium ascorbate, 1 mM cysteine, 1 mM pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. Where applicable, PTH fragments were added for 24 h. Subsequently, the media were exchanged and the cultures were maintained for another 48 h in the presence of 1 µCi

[¹⁴C]proline (uniformly labeled, 285 mCi/mmol, Amersham International) and analyzed for collagen synthesis.

Synthesis of Collagen II and X

The radiolabeled agarose cultures were homogenized and newly synthesized collagens extracted by digestion with pepsin (6). The total extracted proteins were precipitated with ethanol and analyzed by SDS-PAGE (4.5–15% gradient gel) followed by fluorography. All samples corresponded to the same cell number (1.5×10^6 cells). Alternatively, collagen chains separated by SDS-PAGE were subjected to immunoblotting with a polyclonal rabbit antibody, which specifically recognizes human collagen type X (56) and a chemoluminescence detection system (ECL, Amersham International). Duplicates were performed using cells of the same preparation, cultured and analyzed under identical conditions.

Monolayer Cell Culture Conditions

Bovine fetal cartilage (vertex-breech length of the fetuses: ~40–50 cm corresponding to a developmental stage of the second trimester of gestation) was prepared from femoral heads, condyli, and tibial plateaus. A few samples of human fetal epiphyseal cartilage (femur, tibia plateau) were obtained from the Pathology Department of the University of Erlangen-Nürnberg (Erlangen-Nürnberg, Germany). For preparation of chondrocytes, the epiphyseal growth plates were cleaned of perichondrium and dissected from the bony diaphysis, carefully avoiding any loss of cartilage. Subsequently, a 1–2-mm thick layer containing the hypertrophic zone was separated from the rest of the epiphysis by a transverse cut. After dissection each fragment was further processed separately for the isolation of distinct chondrocyte populations representing the phenotype of the hypertrophic and proliferating zones of cartilage.

The tissues slices were minced and treated with 0.1% (wt/vol) Pronase (Boehringer Mannheim, Mannheim, Germany) in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate and finally treated with 0.1% (wt/vol) Clostridial Collagenase P (Boehringer, Mannheim) in Ham's F-12 medium at 37°C for 4–6 h. The tissue fragments were disintegrated mechanically by repeated aspiration with a pipette, and matrix-free cells were passed through three layers of 100 µm pore-size Nylon tissue, recentrifuged, and suspended in an appropriate volume of Ham's F-12 medium. Viability, determined by the trypan blue exclusion technique, always exceeded 90%.

All cell culture experiments were performed under strict serum-free conditions without any undefined additives or substitutes for serum components in Ham's F-12 medium, containing 1 mg/ml pyruvate as an antioxidant (53) and 50 mg/ml ascorbate. The primary cultures of chondrocytes were plated at high densities ($1.3\text{--}1.9 \times 10^5$ cells/cm²) to keep them in their original differentiated phenotype. After the isolation procedure from the cartilage tissue, the cells were given 6 h to recover from the enzymatic treatment before the stimulation experiments with the different PTH fragments were performed.

For in situ hybridization studies of collagen gene expression primary chondrocytes were cultured on tissue culture chamber slides (Nunc Inc., Naperville, IL) at a cell density of 20,000 cells/chamber (8 chambers/slide).

RNA Probes

The specific RNA probes for detection of collagen chains α1(I), α1(II), α1(III), and α1(X) are described in detail in Aigner et al. (2) and Reichenberger et al. (45). Briefly, the pHCG 1N clone contains a 207-bp fragment of the N-propeptide region of human α1(I) and pH CG2 is a 435-bp fragment from the 3' untranslated region of human α1(II). The insert of pH-CG3 is a 294-bp fragment from the C-propeptide domain of human α1(III). The pERX fragment (292 bp) codes for a part of the COOH terminal, nontriple helical domain (NC1) of human collagen type X (45). pRNA 1 contains a XbaI–BamHI fragment (294 bp) of mouse 18S ribosomal RNA (rRNA) from pCM 1 (17) recloned in XbaI–BamI sites of pGEM 3Z. This probe exhibits 100% homology to human 18S rRNA and was used as a positive control and for standardization purposes. The riboprobe for human γ-actin was pHFγ A1 (14).

In Situ Hybridization

All specific cDNA probes for α1(I), α1(II), α1(III), and α1(X), 18S rRNA used in the experiments were cloned into pGEM transcription vectors (Promega Corp., Madison, WI) (2). The constructs were linearized and transcribed in vitro with T7 and SP6 RNA-polymerase (Promega, Madi-

son), to generate anti-sense and sense transcripts, respectively. The probes were labeled with 60–150 μCi (α - ^{35}S)-UTP (1,200 Ci/mmol, New England Nuclear, Dreieich, Germany). The quality of the transcripts was controlled using denaturing formaldehyde agarose and polyacrylamide gels.

In situ hybridization was performed according to Aigner et al. (2) with slight modifications. Briefly, the cultured chondrocytes were fixed on tissue chamber slides in 4% paraformaldehyde in PBS for 4 min at room temperature, dipped in dH₂O and stored in ethanol until use. Cells were rehydrated, treated with proteinase K (20 $\mu\text{g}/\text{ml}$ for 7 min), postfixed, and acetylated in 0.25% acetic anhydride. The subsequent hybridization was performed with riboprobes at a final activity of $3\text{--}6 \times 10^7$ cpm/ml, depending on their length, for 12–16 h at 43°C. After hybridization the specimens were washed in $2 \times \text{SSC}/0.5\%$ β -mercaptoethanol and $0.5 \times \text{SSC}/0.5\%$ β -mercaptoethanol at 40°C and treated with RNases A (20 $\mu\text{g}/\text{ml}$) and T1 (50 U/ml). This treatment was followed by two washes in $2 \times \text{SSC}/50\%$ formamide/ 0.5% β -mercaptoethanol at 45°C and three washes in $0.1 \times \text{SSC}$ at room temperature. Subsequently, the slides were dehydrated and autoradiographed using Kodak NTB-2 Nuclear Track Emulsion (Eastman Kodak Rochester, NY). After a 3–4 d exposure, the slides were developed (Dektol, Eastman Kodak), fixed (Unifix, Kodak), and counterstained in 5% Giemsa. The results were evaluated by dark- and bright-field microscopy (Zeiss).

Northern Blot

Chondrocytes were lysed in 4 M guanidinium thiocyanate, 25 mM Na-citrate, 0.5% Na-sarcosyl, and 0.7% β -mercaptoethanol. RNA was extracted using the cesium-chloride density centrifugation method of Chirgwin et al. (9). Total RNA (10 μg) was subjected to formaldehyde gel electrophoresis, blotted onto nylon filters and cross-linked by exposure to UV light for 5 min. For analysis of RNA, cDNA probes were labeled with [^{32}P]dATP or dCTP by random priming and hybridized in 50% formamide, $5 \times \text{SSC}$, $5 \times$ Dehardt's solution (4), 0.5% SDS, and 100 $\mu\text{g}/\text{ml}$ herring sperm DNA at 42°C for 16 h. After hybridization, filters were washed twice in $2 \times \text{SSC}$ at room temperature for 5 min, twice in $2 \times \text{SSC}/0.1\%$ SDS each at 50°C for 30 min, and once in $0.1 \times \text{SSC}/0.1\%$ SDS at room temperature for 2 min. The washed filters were exposed to Kodak X-OMATTM X-ray films (Eastman Kodak).

Microfluorometry of Ca^{2+} -Signaling

For Ca^{2+} imaging, chondrocytes were seeded on 35-mm tissue culture dishes and incubated in Ham F-12 for 24 h before loading with Fura-2/AM (5 μM) for 30 min at 37°C. Ca^{2+} was imaged with an upright microscope (Zeiss Axioskop FS, Jena, Germany) and a $40\times$ water immersion objective. A CCD camera system (Photometrics Ltd., Tuscon AZ) (12, 37, 38) was used to acquire digitized images of Fura-2 fluorescence. Free Ca^{2+} concentrations were determined from background corrected image pairs at 350 and 380 nm excitation with the ratio method (18). The responsiveness of the calcium signaling machinery of the chondrocyte population was controlled by the Ca^{2+} response to 5 μl FCS. Subsequently, the return of intracellular free Ca^{2+} to stable baseline levels was recorded for at least 10 min before the application of PTH fragments. Cells were continuously superfused with saline containing NaCl 140 mM, KCl 5 mM, MgSO_4 2 mM, NaH_2PO_4 1 mM, glucose 5.5 mM, Hepes 20 mM, pH 7.4. The PTH peptides were applied by microdrop application of concentrated stock solution into the bath to give final concentrations as indicated. When different PTH fragments were sequentially tested on the same chondrocyte population, a return to baseline Ca^{2+} fluorescence and stability for 10 min was imperative before application of a new peptide.

Results

Stimulation of Collagen Types II and X Protein Synthesis by Human Costal Chondrocytes

A mixture of resting, proliferative, and hypertrophic human costal chondrocytes was cultured in agarose gels under serum-free conditions (53). To investigate the effect of PTH fragments from the amino-terminal (PTH (1-34))- and the carboxyl-terminal (PTH (52-84)) end of the hormone on collagen biosynthesis, the cultures (cell density: $2 \times 10^6/\text{ml}$) were exposed to the PTH peptides at 10^{-8} M

for 24 h. Subsequently, the cultures were labeled with radioactive proline for 48 h. Pepsin-treated collagens from the culture dishes were isolated and analyzed by SDS-PAGE and fluorography. In comparison to the serum-free control (Fig. 1 a, lane a), biosynthesis of collagen II was enhanced under the influence of the NH₂- and COOH-terminal PTH fragments (Fig 1a, lanes b and c). Collagen X was not detectable by fluorography due to the low ratio of type X to type II collagen in the mixed chondrocyte population, but was evident after a longer exposure of the gel (not shown). The synthesized type X collagen was analyzed by immunoblotting of the same sample with a type X collagen-specific antibody. As shown in Fig. 1 b, collagen X synthesis was not detectable in controls (lane a), but was prominent after stimulation with either PTH (1-34) (lane b) or PTH (52-84) (lane c). Thus, these experiments demonstrate a stimulatory effect of PTH peptides on the synthesis of collagens II and X by human postnatal chondrocytes under serum-free cell culture conditions.

Since human costal chondrocytes represented a mixture of resting, proliferative, and hypertrophic chondrocytes,

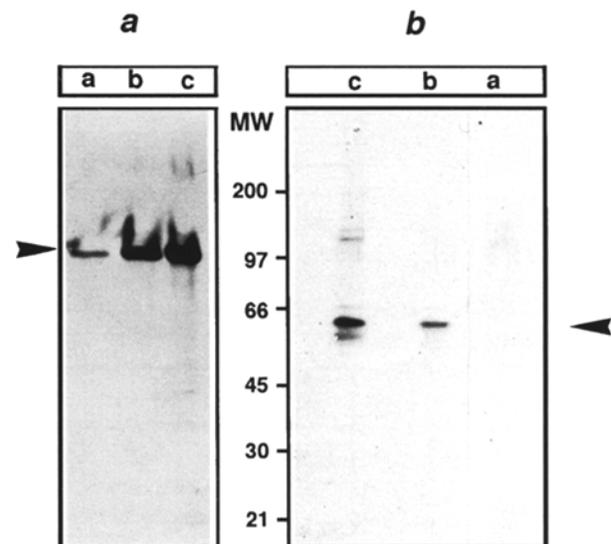


Figure 1. Stimulation of collagen types II and X protein synthesis in postnatal human costal chondrocytes in agarose culture by PTH fragments. (a) Collagen type II synthesis determined by incorporation of [^{14}C]proline: Cultures were established under serum-free conditions at a density of 2×10^6 cells/ml in 0.75 ml of agarose gels, cultured and labeled with [^{14}C]proline for 48 h. The medium (DMEM) was supplemented with PTH (1-34) (lane b) or PTH (52-84) (lane c) to a final concentration of 10^{-8}M . Collagens from agarose and medium were pepsin treated and analyzed by SDS-PAGE (4.5–15% gradient gel) and fluorography. The PTH-free medium control is shown in lane a. At the short exposure time (2 d) only the dominating $\alpha 1(\text{II})$ -band is visible (arrowhead: $\alpha 1(\text{II})$.) (b) Collagen type X synthesis determined by immunoblotting with a collagen X specific rabbit antiserum: In parallel, chondrocytes from the same preparation were cultured under conditions identical to those described in a but without [^{14}C]proline labeling. Pepsinized collagens were harvested and electrophoretically separated as in Fig. 1 a, blotted to a nitrocellulose filter and stained with a specific rabbit anti-collagen x: lane a is the medium control; lane b: PTH (1-34); and lane c: PTH (52-84) (arrowhead: $\alpha 1(\text{X})$.)

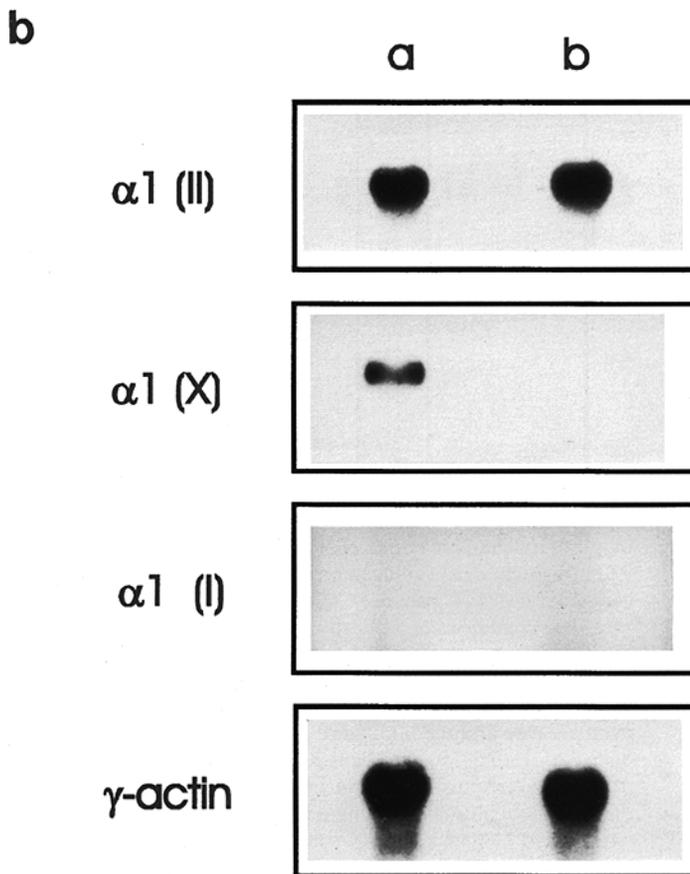
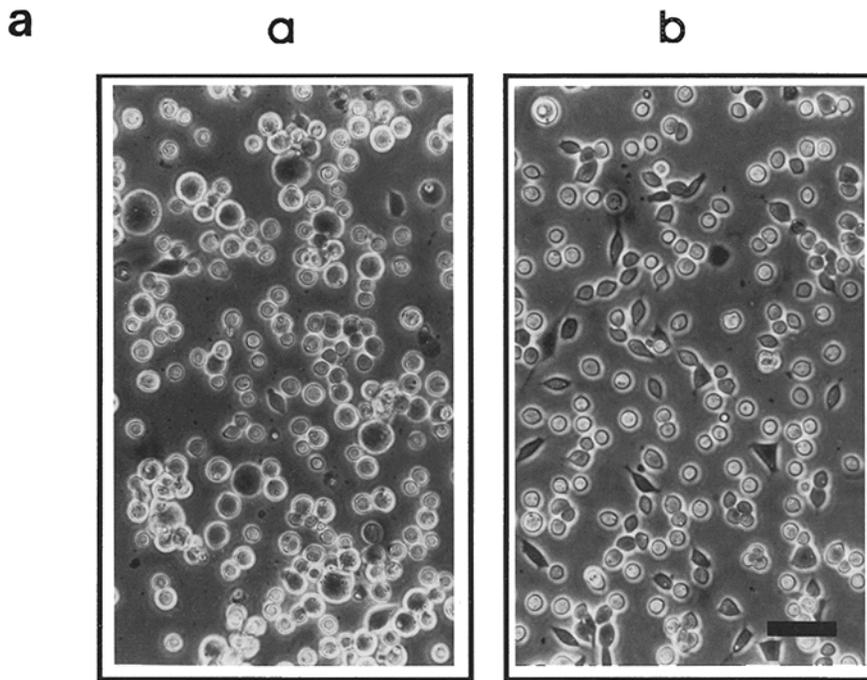


Figure 2. Characterization of the differentiation stage of the chondrocytes. (a) Phase contrast microscopy of the chondrocyte preparations from bovine fetal epiphyseal cartilage representing the population from the hypertrophic zone (a) and the resting and proliferating zone (b). (b) Northern blot analysis. RNA was extracted from the hypertrophic chondrocytes of the growth plate (a) and proliferating chondrocytes (b) freshly isolated under serum-free conditions. Equal amounts of total RNA (10 μ g) were applied to each lane. The blot was hybridized sequentially with the human riboprobes for collagen type II, type X, type I, and γ -actin. A strong band for collagen type II (exposure: 1 d) and γ -actin (exposure: 2 d) is visible (lanes a and b). After 3 d of exposure a 3.0-kB band of type X collagen appeared in lane a. No mRNA is detectable for type X collagen in lane b, and for type I collagen in both lanes, even after 7 d exposure. Bar, (a in panel b) 33 μ m.

we determined the differential effect of the NH₂- and COOH-terminal PTH fragments on proliferating vs hypertrophic chondrocytes. Subsequent studies were performed on separate preparations of chondrocytes from the

resting zone and a population enriched in hypertrophic chondrocytes, isolated from bovine cartilage. Fetal bovine chondrocytes were chosen because of their availability and their physiological similarity to human chondrocytes.

Phenotypic Characterization of the Chondrocyte Populations from the Proliferative and Hypertrophic Zone of Fetal Bovine Epiphyseal Cartilage

Care was taken to separate proliferating from hypertrophic chondrocytes during the isolation of the cells from fetal bovine epiphyseal cartilage. This procedure resulted in two distinct chondrocyte populations. Their phenotypic characterization 24 h after preparation from cartilage is shown in Fig. 2. The cell population from the hypertrophic zone of cartilage was enriched in enlarged cells (Fig. 2 *a*); Northern blotting revealed expression of type II as well as type X collagen mRNA (Fig. 2 *b*) at a mean ratio of 8:1 (variation between 5:1 and 14:1) as assessed by densitometric analysis in four independent preparations. In contrast, no expression of collagen type X was detectable in

the cell population prepared from the resting zone of epiphyseal cartilage. A significant contamination of the primary cultures with fibroblasts or dedifferentiated chondrocytes was excluded, since Northern blot analysis for collagen type I expression remained negative immediately after the cell preparation procedure from the tissue (Fig. 2 *b*) and throughout the subsequent culture period.

In Situ Hybridization on Monolayer Cultures of PTH-stimulated Fetal Chondrocytes from the Proliferative Zone of the Epiphysis

Monolayer cultures of freshly isolated fetal human and bovine chondrocytes from the proliferating zones of the epiphysis were incubated with varying concentrations (10^{-6} – 10^{-11} M) of PTH (1-34) and PTH (28-48) under

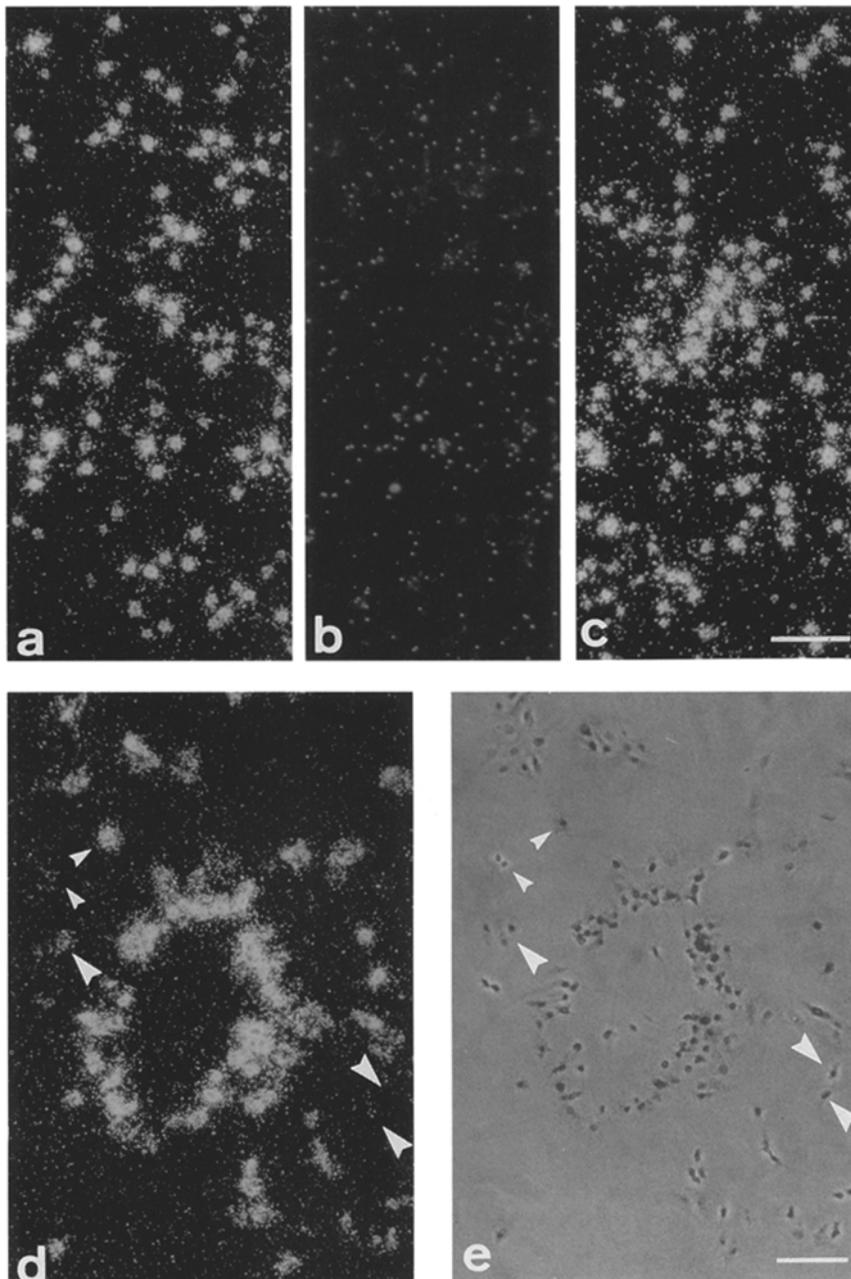


Figure 3. In situ hybridization with a riboprobe specific for $\alpha 1(\text{II})$ mRNA. Primary monolayer cultures of human fetal epiphyseal chondrocytes were incubated for 24 h in (a) 10^{-8} M PTH (1-34), (b) Ham's F-12 medium (c) medium supplemented with 10% FCS, and (d) 10^{-8} M PTH (28-48) (dark field). At a higher magnification and by comparison with the bright field (e), the heterogeneity of collagen type II expression in the cell population is visible and indicated by the arrows. Bars: (c) 66 μm ; (e) 26 μm .

strict serum-free conditions on tissue chamber slides for 24 h. Subsequently, in situ hybridizations were performed on the specimens using RNA probes for $\alpha 1(\text{I})$, $\alpha 1(\text{II})$ and $\alpha 1(\text{III})$ mRNA to analyze the PTH effect on collagen gene expression. The use of chamber slides allowed for analysis of control cells after incubation in serum-free Ham's F-12 medium alone, or in medium supplemented with 10% FCS in different chambers on the same slide.

Both PTH (1-34) and PTH (28-48) stimulated $\alpha 1(\text{II})$ expression in the chondrocytes at concentrations between 10^{-6} – 10^{-10} M. At a concentration of 10^{-8} M the effect of the PTH fragments on collagen II expression reached an optimum, which was quantitatively comparable to the stimulation obtained by FCS.

The results were evaluated semiquantitatively in representative areas by counting cells with unequivocal positive signals and by counting grains per cell. Representative areas of human monolayer cultures after 24 h exposure to medium alone, PTH (1-34) (10^{-8} M), PTH (28-48) (10^{-8} M), or FCS are shown in Fig. 3. A stimulatory effect was obtained by evaluation of at least three parallel experiments in three independent chondrocyte preparations from different sources. This effect was specific for collagen type II, as no inductive effect on $\alpha 1(\text{I})$ or $\alpha 1(\text{III})$ gene expression in the chondrocytes was detectable (data not shown). These results were reproducible in four independent experiments with bovine fetal chondrocytes. However, Fig. 3 also demonstrates the heterogeneity of the response in these primary cultures of fetal chondrocytes. A precise quantitative analysis of the in situ hybridization experiments was not possible. It was therefore necessary to confirm these observations by Northern blot analysis.

Northern Blot Analysis of PTH-stimulated Proliferating Chondrocytes

Quantitative analysis of $\alpha 1(\text{II})$ expression in proliferating bovine fetal chondrocytes was done by Northern blot. Fig. 4 *a* shows $\alpha 1(\text{II})$ signals after incubation of the cells with PTH (1-34) and PTH (28-48) at concentrations of 1 and 10 nM, respectively, for 24 h. Both fragments exhibited specific stimulatory effects on collagen type II mRNA levels without affecting the expression of $\alpha 1(\text{I})$, $\alpha 1(\text{III})$, or $\alpha 1(\text{X})$. This stimulatory effect was more pronounced at an effector concentration of 10 nM.

Since both PTH peptides were active, we questioned whether the effect was mediated by different functional domains on each fragment or rather by a common effector site located in the overlap region (aa 28 - 34) of their sequences. As the NH_2 -terminal two amino acid residues of PTH are essential for the activation of adenylate cyclase (43, 52), we performed an inhibition experiment with a known receptor competitor for PTH (1-34) lacking the critical functional domain for cAMP induction at its NH_2 terminus. The result of this inhibition experiment is shown in Fig. 4 *b*. Even in a 1,000-fold molar excess, the inhibitor failed to exert any effect on the stimulation of $\alpha 1(\text{II})$ expression by PTH (1-34), indicating that the functional domain is not located at the NH_2 terminus and that cAMP induction is not critically involved as a second messenger.

In contrast, the competitor fragment PTH (3-34) stimulated $\alpha 1(\text{II})$ expression in chondrocytes. To map the effec-

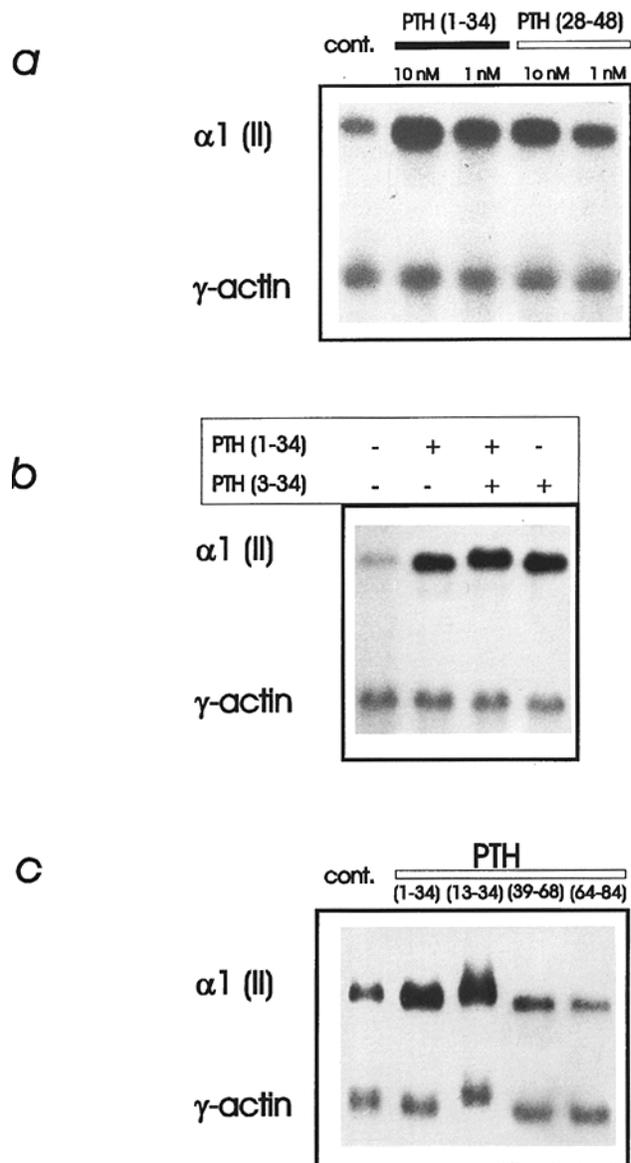


Figure 4. Northern blot analysis of $\alpha 1(\text{II})$ expression in bovine fetal chondrocytes. Fetal bovine proliferating chondrocytes in monolayer culture were stimulated with different PTH fragments under serum-free conditions for 24 h. Each lane was loaded with 10 μg of total RNA. The blot was hybridized sequentially with the human riboprobe for collagen type II and γ -actin. (a) Stimulatory effect of PTH (1-34) and PTH (28-48) on $\alpha 1(\text{II})$ mRNA expression: the chondrocytes were incubated with PTH (1-34) and PTH (28-48) at a concentration of 10^{-8} M and at 10^{-9} M, respectively. cont., medium control. (b) Inhibition experiment of the stimulatory effect of PTH (1-34) by a receptor antagonistic peptide (PTH (3-34)) for cAMP dependent responses: Chondrocytes in monolayer culture were stimulated with PTH (1-34) at a concentration of 10^{-9} M as in 4 *a*, but in the presence of the antagonist PTH (3-34) at a concentration of 10^{-6} M (lane 3). Lane 1 is the medium control and lane 2 the stimulation of $\alpha 1(\text{II})$ mRNA by PTH (1-34) alone. Lane 4 shows the result of the control for an agonistic activity of PTH (3-34) alone (concentration: 10^{-8} M). No inhibition by the receptor competitor PTH (3-34) is detectable. (c) Stimulatory effect of different PTH peptides on $\alpha 1(\text{II})$ mRNA expression. Medium control is shown in lane 1 (cont.). All fragments were tested at a concentration of 10^{-8} M: PTH (1-34) (lane 2), PTH (13-34) (lane 3), PTH (39-68) (lane 4), and PTH (64-84) (lane 5).

tor domain on the PTH fragments more precisely, additional peptides were tested for their stimulatory potential on collagen type II expression (Fig. 4 c). PTH fragments covering the COOH-terminus of the hormone PTH (39-68), PTH (52-84) (result not shown), and PTH (64-84) were inactive, whereas PTH (13-34) was proven to harbor the effector domain.

Densitometric evaluation of a series of stimulation experiments with the different PTH fragments revealed that the agonistic peptides of the central portion of PTH induced an approximately fivefold increase in $\alpha 1(\text{II})$ expression over base line levels of the medium controls (Fig. 5 a).

In Fig. 5 b an alignment of the different PTH peptides along the entire hormone sequence is shown together with a summary of their effects on collagen type II expression

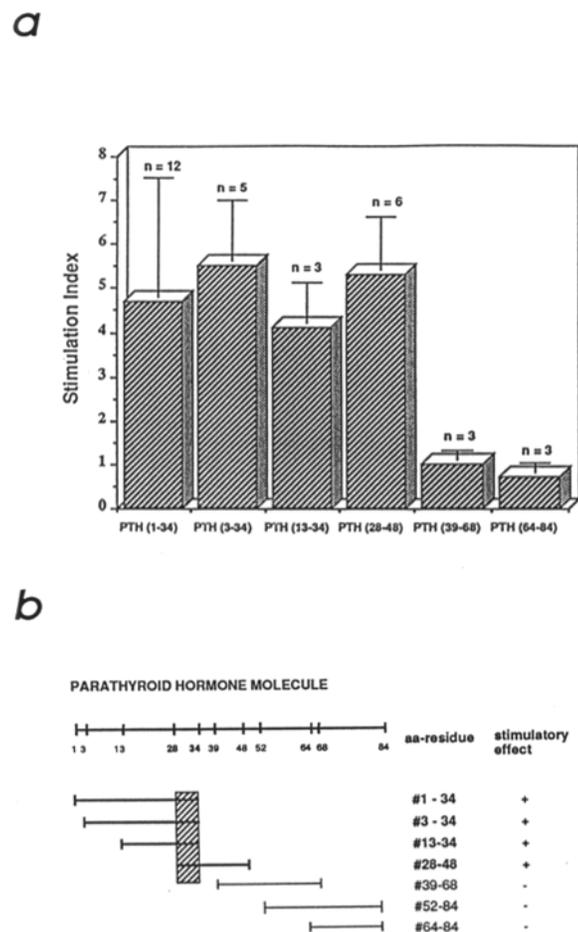


Figure 5. (a) Densitometric analysis of Northern blots from independent stimulation experiments (n) of proliferating bovine chondrocytes with the different PTH fragments (concentration 10^{-8} M): PTH (1-34), PTH (3-34), PTH (13-34), PTH (28-48), PTH (39-68), and PTH (64-84). The columns represent the stimulation indices for $\alpha 1(\text{II})$ mRNA levels, which were determined after normalization with γ -actin mRNA and $\alpha 1(\text{II})$ mRNA of unstimulated controls. Error bars represent standard deviation. (b) Summary of the effects of different PTH peptides on $\alpha 1(\text{II})$ expression in young proliferating chondrocytes. The hatched area, corresponding to amino acid residues 28-34 of the PTH molecule, indicates a functional domain which is present in the sequences of all agonistic fragments (**bold**) and is absent from the inactive peptides. (# = PTH fragment.)

in proliferating bovine fetal chondrocytes. All functionally active fragments overlap in the central region spanning aa residues 28-34, which is not present in the inactive fragments from the COOH-terminal portion of PTH. Therefore, this domain seems to be essential for the stimulation of $\alpha 1(\text{II})$ expression.

PTH Effects on Chondrocyte Cultures Established from Hypertrophic Cartilage

Based on the results of effector domain mapping in experiments with proliferating chondrocytes, PTH (1-34) was chosen as an appropriate fragment for analogous stimulation experiments in monolayer cultures of chondrocytes from the hypertrophic zone of fetal bovine cartilage. Also, in chondrocyte cultures enriched in hypertrophic cells, PTH (1-34) stimulated type II collagen expression at a concentration of 10 nM (Fig. 6, a and b). In addition, a stimulation of $\alpha 1(\text{X})$ expression was seen, whereas the Northern blots remained negative with $\alpha 1(\text{I})$ and $\alpha 1(\text{III})$ specific riboprobes (results not shown). Moreover, in contrast to the results obtained with resting and proliferating chondrocytes, an additional stimulatory effect of the COOH-terminal part of PTH (PTH (52-84)) on type II and type X collagen gene expression was observed (Fig. 6, a and b). Densitometric evaluation of the Northern blots revealed a fivefold increase in $\alpha 1(\text{II})$ or $\alpha 1(\text{X})$ mRNA levels in chondrocytes from the hypertrophic zone in response to stimulation with either PTH (1-34) or PTH (52-84) (Fig. 6 b).

As the chondrocyte preparations from the hypertrophic zone of cartilage were to some extent heterogeneous (Fig. 2 a), the PTH stimulation experiments were repeated with a population of chondrocytes clearly dominated by cells of the characteristic hypertrophic phenotype (Fig. 7 a). The results from these experiments revealed a stimulatory effect of PTH (28-48) and confirmed the initial data on the effect of PTH (1-34) and PTH (52-84) on $\alpha 1(\text{II})$ and $\alpha 1(\text{X})$ mRNA expression (Fig. 7 b).

These results demonstrate the importance of the COOH-terminal part of PTH for collagen mRNA expression in hypertrophic chondrocytes, in addition to the functional domain in the central region of the hormone, which also acts on proliferating chondrocytes.

The responsiveness of the chondrocytes to the stimulatory effects of PTH fragments on $\alpha 1(\text{II})$ and $\alpha 1(\text{X})$ mRNA expression is not a transient phenomenon restricted to the 6-h period which was usually given to the cells to recover from the enzyme treatment during the separation procedure from the tissue. The Northern blot in Fig. 8 shows that cells after 3 d culture in serum-free medium still responded to PTH stimulation with an increase in $\alpha 1(\text{II})$ mRNA.

Intracellular Free Ca^{2+} after Stimulation with Different PTH Peptides

For the analysis of cAMP independent second messenger signals, monolayers of bovine chondrocytes from the hypertrophic zone were loaded with Fura-2/AM and stimulated with different PTH fragments. The PTH peptides were selected on the basis of the Northern blot results according to their ability to exert a stimulatory effect on collagen gene expression. Therefore, PTH (1-34), PTH (28-48), and

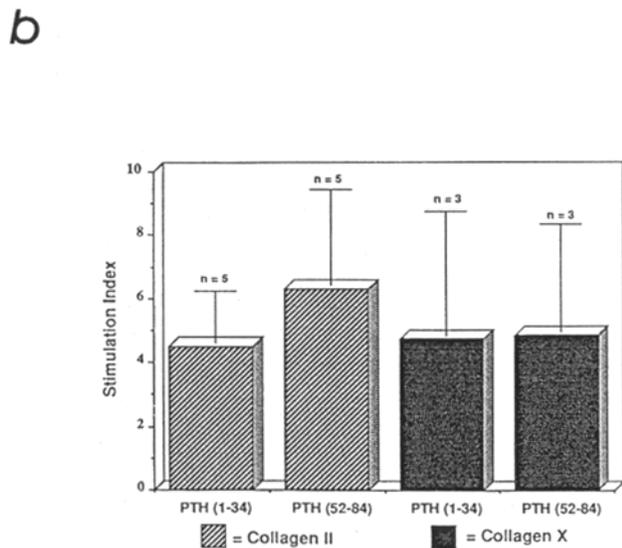
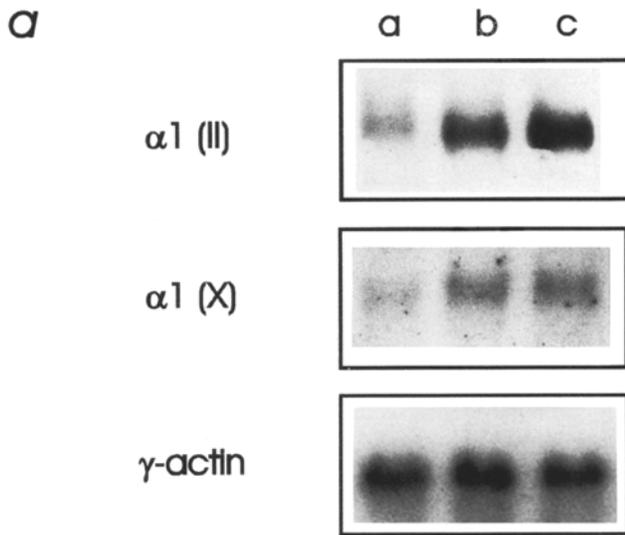


Figure 6. (a) Northern blot analysis of $\alpha 1(\text{II})$ and $\alpha 1(\text{X})$ expression in chondrocytes from the growth plate of bovine fetal cartilage. Monolayer cultures of hypertrophic chondrocytes were stimulated with the hormone fragments PTH (1-34) (lane b) and PTH (52-84) (lane c) under serum-free conditions for 24 h. Medium control is shown in lane a. Each lane was loaded with 10 μg of total RNA. The blot was hybridized sequentially with riboprobes for $\alpha 1(\text{X})$, $\alpha 1(\text{II})$, and γ -actin. (b) Densitometric analysis of independent stimulation experiments (n) of chondrocytes from the hypertrophic zone of bovine growth plate cartilage with PTH (1-34) and PTH (52-84). The columns represent the stimulation indices for $\alpha 1(\text{II})$ and $\alpha 1(\text{X})$ levels, which were determined by normalization with γ -actin mRNA and $\alpha 1(\text{II})$ or $\alpha 1(\text{X})$ mRNA in unstimulated controls, respectively. Error bars represent standard deviation.

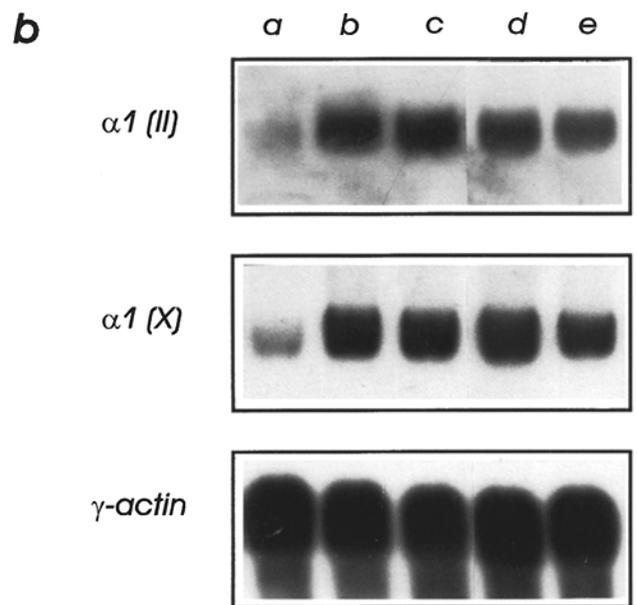
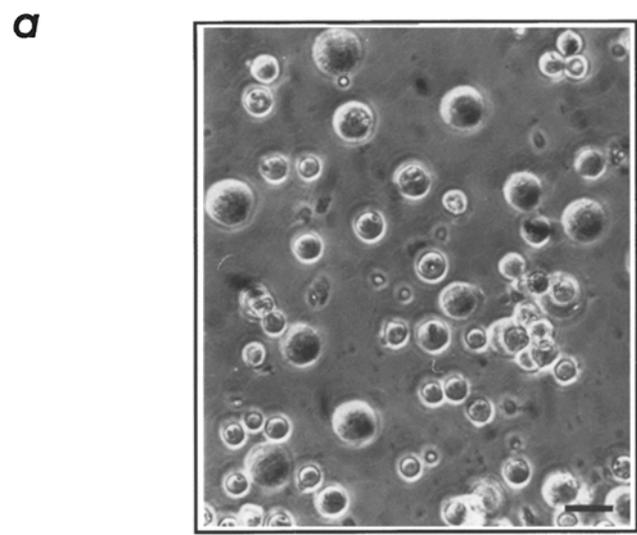


Figure 7. (a) Phase contrast microscopy of chondrocyte preparations especially enriched in hypertrophic chondrocytes. (b) Northern blot analysis of $\alpha 1(\text{II})$ and $\alpha 1(\text{X})$ expression in chondrocyte populations enriched in hypertrophic cells (a). Monolayer cultures were stimulated with the hormone fragments PTH (1-34) (lane b), PTH (28-48) (lane c), and PTH (52-84) (lane d) under serum-free conditions and with 10% FCS (lane e) for 24 h. Medium control is shown in lane a. Each lane was loaded with 10 μg of total RNA. The blot was hybridized sequentially with riboprobes for $\alpha 1(\text{X})$, $\alpha 1(\text{II})$, and γ -actin.

PTH (52-84) were further studied for their ability to induce changes of intracellular free Ca^{2+} concentrations by ratio imaging of up to 20 chondrocytes simultaneously in independent experiments from five different preparations of chondrocytes (Table I). For the investigation of Ca^{2+} signaling, primary cultures of fetal chondrocytes were used. In a series of experiments the different PTH peptides were applied sequentially on the same chondrocyte population. After application of one peptide a return to stable intracellular baseline calcium levels was recorded

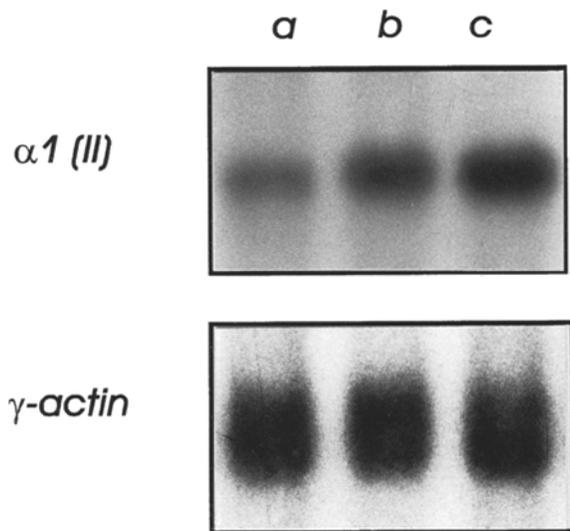


Figure 8. Northern blot analysis of $\alpha 1(\text{II})$ expression in chondrocyte populations enriched in hypertrophic cells. The cells were kept for 3 d in serum-free Ham's F-12 medium after preparation from growth plate cartilage. Subsequently, the cells were stimulated for 24 h with PTH (1-34) (lane b), or PTH (52-84) (lane c), and medium control (lane a).

for at least 10 min before a new PTH fragment was tested. All fragments were capable of inducing a transient increase of intracellular free calcium within 5 min of application of the stimulus (Fig. 9 and Table I). Normally, the Ca^{2+} concentration returned to stable baseline levels (see controls in Fig. 9, a and b). In a few cells we observed secondary and tertiary increases in intracellular Ca^{2+} at intervals of 2–5 min. Fig. 9 a shows that within one cell population some cells responded exclusively to PTH (1-34), while others responded only to PTH (52-84). Some cells exhibited a promiscuous calcium response and were sensitive to both fragments. This simultaneous responsiveness to PTH (1-34) and PTH (52-84) (Fig. 9 a) may differ from the cellular response of some chondrocytes to the PTH fragments 1-34 and 28-48 (Fig. 9 b) with regard to differential receptor involvement. Thus, the NH_2 -terminal PTH peptide does not exhibit any sequence overlap with PTH (52-84). In contrast, PTH (1-34) and PTH (28-48) share a conserved stretch of amino acids between aa 28-34 and may exert their effect on intracellular Ca^{2+} via this common functional domain.

For comparison, a pure chondrocyte preparation from the zone of resting and proliferating cartilage was analyzed with the result that only 3 out of 150 FCS-responsive cells showed an increase in intracellular free Ca^{2+} in response to the COOH-terminal fragment (vs 35 out of 119 in the chondrocyte population from the hypertrophic zone, Table I). However, the method selects for a subset of cells with the ability to firmly adhere to the bottom of the plastic dish in order to allow for Ca^{2+} measurements under a continuous flow of medium. This explains why the cells in Fig. 9 differ in cell shape from those in Fig. 2. Therefore, the number of positive Ca^{2+} responses to different PTH stimuli does not necessarily represent the PTH-sensitive cells in the total population of hypertrophic or proliferating chondrocytes. However, the experiments demonstrate

the potential of all PTH peptides to induce increases in free Ca^{2+} in the cells and a differential responsiveness of individual chondrocytes towards different PTH fragments.

Discussion

The results presented in this study show that different fragments of the PTH molecule stimulate collagen type II and X gene expression in chondrocytes under serum-free conditions. In initial experiments using postnatal human costal chondrocytes, PTH (1-34) and PTH (52-84) stimulated synthesis of collagen type II and X in a serum-free agarose culture system. Due to the restricted availability of appropriate amounts of human chondrocytes and the disadvantages of the agarose culture system for studies of gene expression at the mRNA level, further experimentation was performed using a serum-free culture system of bovine growth plate chondrocytes, separated into cells from the resting and proliferating zone, and in cells from the hypertrophic zone. In agreement with the stimulation of collagen type II and X at the protein level, a rise in $\alpha 1(\text{II})$ and $\alpha 1(\text{X})$ mRNA level was detected in response to different PTH peptides. The results clearly show that the stimulatory PTH effect on collagen mRNA levels is dependent on the differentiation stage of the cells and induced by at least two different functional domains of PTH. The first domain is located in the central part of the PTH molecule between aa 28-34 and is capable of stimulating $\alpha 1(\text{II})$ expression in resting and proliferating fetal chondrocytes. A second domain is located in the COOH-terminal part of PTH between aa 52-84. This domain is recognized only by cells which are differentiating towards the hypertrophic stage; it is not active on proliferating chondrocytes. Fragments lacking the genuine NH_2 -terminus (aa residues 1-3) of the hormone, which is indispensable for activation of the PTH receptor associated adenylate cyclase (15, 43, 52) also stimulate type II collagen expression. This indicates that cAMP does not play a critical role in the signaling pathway of PTH-mediated upregulation of type II collagen gene expression.

All functionally active fragments are capable of inducing a rise in calcium concentration in the chondrocytes as shown in Fig. 9. Similarly, a cAMP independent Ca^{2+} -signaling, involving protein kinase-C activation (57) has been demonstrated in other experimental systems of PTH action (48). The activation of this signal transfer cascade by PTH is dependent on a region (aa 28-34) in the central part of PTH (23). We mapped the functional domain for stimulation of collagen type II gene expression in proliferating chondrocytes to the same region. It is, therefore, very likely that a Ca^{2+} signal induced by the protein kinase-C domain of PTH (23) is also involved in the upregulation of $\alpha 1(\text{II})$ expression in proliferating chondrocytes.

In chick chondrocytes, it has been shown (48) that this central PTH domain mediates an EGTA-sensitive mitogenic effect on the cells. However, under the experimental conditions of this study which are high plating density of the cells ($1.3\text{--}1.9 \times 10^5/\text{cm}^2$), a 24 h period of hormone treatment, and strict serum-free conditions, no mitogenic effect was detectable for any PTH fragment. These culture conditions account for the absence of any proliferative re-

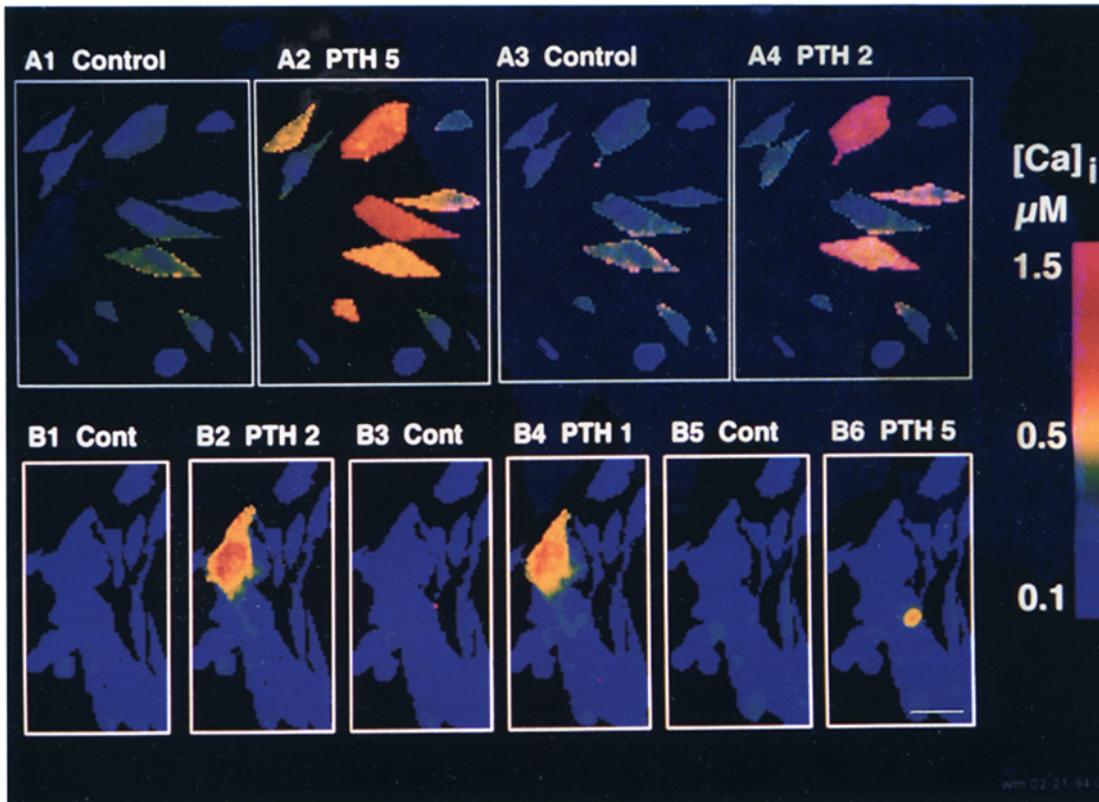


Figure 9. Ratio imaging of intracellular free calcium in bovine fetal chondrocytes as determined by Fura-2 fluorescence. Monolayer cultures of chondrocytes from the hypertrophic zone were stimulated by a bolus application (final concentration: 10^{-8} M) of PTH (52-84) (A2 PTH5 and B6 PTH5), PTH (28-48) (A4 PTH2 and B2 PTH2), or PTH (1-34) (B4 PTH1). *A* shows representative ratio images obtained by a stimulation experiment with a sequential application of PTH peptides 52-84 and 28-48. A1 is the control image before stimulation with PTH (52-84). A3 shows the return to a stable baseline level and represents the control image preceding stimulation with PTH (28-48). *B* shows the images derived from a sequential stimulation experiment with three different PTH fragments: PTH (28-48) (B2 PTH2), PTH (1-34) (B4 PTH1), and PTH (52-84) (B6 PTH5). B1 Cont is the control image before stimulation; B3 Cont and B5 Cont demonstrate the return to stable baseline after the preceding stimulations with the respective PTH peptides. Bar, 20 μ m.

sponse of the chondrocytes to PTH, which is in accordance with the observations made by Schlüter et al. (48) that high cell densities impede the mitogenic effect of PTH. Therefore, the same functional domain of PTH (amino acid residue 28-34) can exert quite different effects, either a mitogenic response or an increase in differentiated function (α 1(II) expression), depending on the duration of the hormonal stimulus and the cell density.

Table 1. Ca^{2+} Response Frequency in Fetal Bovine Chondrocyte from the Hypertrophic Zone of the Epiphysis: Ratio Image Analysis

	PTH (1-34)	PTH (28-48)	PTH (52-84)	FCS
Responses (<i>n</i>)	19	24	35	127
Total cells (<i>n</i>)	50	85	119	150
Response frequency (%)	26.0	28.2	29.4	84.6

Fura-2 loaded chondrocytes from the hypertrophic zone of cartilage were examined by microfluorometry. Changes of intracellular free Ca^{2+} were determined in individual cells by ratio image analysis. A response was considered to have occurred when at least an increase of 0.5 μ M from the baseline Ca^{2+} was recorded. The number (*n*) of responsive cells upon stimulation by different PTH peptides and with FCS is shown. The response frequency was calculated from the ratio of responsive cells to total cells measured.

These environmental influences are critical for PTH-mediated effects, hence differences in culture conditions may also account for some apparently controversial results found in the literature on the hormone action on collagen synthesis by chondrocytes (13, 21, 36, 41). In the study of Crabb et al. (13) articular chick chondrocytes remained totally unresponsive to PTH, whereas a mitogenic effect and inhibition of collagen synthesis was reported in growth plate chondrocytes. Similar inhibitory effects of PTH (1-34) and PTH (54-84) on collagen X synthesis by hypertrophic rabbit chondrocytes were recently reported by Iwamoto et al. (21). In a detailed analysis of PTH effects on long term cultures of maturing chick sternal and tibial growth plate chondrocytes, Iwamoto et al. (21) demonstrated that PTH has an inhibitory effect on the emergence of collagen X expressing cells and that this inhibitory effect persisted for the whole maturation pathway of the chondrocytes, which is in contrast to the more stage-specific effects of FGF-2 (22).

However, these experiments were performed in long term cultures and in the presence of 5–10% FCS in the culture medium, while all stimulatory effects of the PTH fragments on collagen gene expression reported here depend on strict serum-free conditions. We have shown that FCS dramatically enhances collagen gene expression within 24 h.

As uncontrolled effects by endogenous growth factors in FCS cannot be excluded (10), serum was omitted from all stages of the experiments reported here. In agreement with Iwamoto et al. (21, 22) we found in our culture system that the stimulatory PTH (1-34) effect on collagen gene expression was not only abolished, but also reverted, when the chondrocytes were exposed to serum during collagenase digestion before the PTH stimulus (Vornehm, S., manuscript in preparation). Reduced viability of the chondrocytes prepared and cultured under serum-free conditions was excluded by the fact that freshly prepared cells showed strong signals for $\alpha 1(\text{II})$ mRNA and divided normally. Since prolonged enzymatic digestion of cartilage in the absence of serum might cause cell damage, care was taken to reduce the time of enzyme treatment to a minimum. Properly treated chondrocytes remained viable and retained their FCS-responsiveness, as well as responsiveness to PTH. This PTH response of freshly isolated cells remained stable for a culture period of at least 3 d under strict serum-free conditions, however, preference was given in this study to an immediate stimulation of the chondrocytes already 6 h after isolation in order to exclude any uncontrolled influence of *in vitro* (de)differentiation.

The stimulatory effect of PTH peptides on collagen types II and X expression is not restricted to mRNA levels and monolayer conditions; identical results were obtained at protein level with human costal chondrocytes cultured in agarose suspension. Thus, it is likely that the apparent conflict between our data and those published by Iwamoto et al. (21, 22) result from different chondrocyte culture systems and reflect the biologically relevant sensitivity of PTH effects to modulation by growth factors present in serum. Analysis of serum factors that modify the PTH effect would help in the understanding of the complex regulation of collagen metabolism during endochondral bone formation.

In this study a new effector domain for chondrocytes was localized in the COOH-terminal region of PTH (amino acid residues 52-84), which exerts a selective effect on collagen type II and X expression in growth plate chondrocytes from the hypertrophic zone. Proliferating chondrocytes did not respond to PTH peptides derived from the COOH terminus. It has been shown by Murray et al. (40) that cells differentiated towards the osteoblastic lineage (human osteosarcoma SaOS-2 cells) increase type I collagen mRNA levels in response to PTH (1-34), but not to PTH (53-84), although the COOH-terminal fragment stimulated expression of mRNA for osteocalcin, the vitamin D receptor and alkaline phosphatase in the same cells. This underlines the domain specificity and differentiation stage-dependency of the PTH action and supports the concept of a physiological role for PTH metabolites in the hormonal control of matrix metabolism in the growth plate. In accordance with this hypothesis are results from *in situ* hybridization studies on fetal rat cartilage (32), showing strong PTH receptor gene expression in a distinct zone of maturing chondrocytes immediately above the layers of hypertrophic cartilage. Moreover, by light microscope autoradiography, Barling and Bibby (5) demonstrated [^3H]PTH binding to hypertrophic chondrocytes; a histological study from 1943 revealed hypertrophy, calcification, and premature closure of the growth plate induced by intraperitoneal administration of PTH to growing mice (50). In an organ

culture system of mandibular explants, the COOH-terminal fragment PTH 53-84 exerted a profound change of morphology in the zone of hypertrophic cartilage (51).

In this respect our results suggest that one important facet of PTH action is the stimulation of collagen type X gene expression in the hypertrophic zone of the epiphysis. Moreover, modulation of collagen metabolism could be a critical event in calcification of growth plate cartilage since recent data (27, 28, 29) indicate that the interaction of collagen type II and X with the matrix vesicles in the growth plate activate Ca^{2+} loading of these extracellular microstructures, which are considered the initiation sites of mineral deposition in cartilage.

Another aspect of the role of PTH in cartilage mineralization is closely related to our finding that PTH metabolites are capable of inducing a rise in intracellular free Ca^{2+} in chondrocytes from the hypertrophic zone. Matrix vesicles are formed in chondrocytes by budding from the cytoplasmic membrane (3, 19) leaving the possibility open that they retain the chondrocytic PTH receptors in their cell membrane. For mineralization of cartilage, it remains to be elucidated whether the matrix vesicles still respond to PTH fragments after deposition in the extracellular matrix by increasing the intravesicular Ca^{2+} concentration.

It is not yet clear how the COOH-terminal part of PTH is recognized by the hypertrophic chondrocytes, and why the proliferating chondrocytes remain unresponsive to COOH-terminal PTH fragments. PTH and PTH-related peptide (PTHrP) bind to a common heptahelical G-protein coupled receptor molecule (24, 47). Since this classical PTH receptor has a widespread tissue distribution, receptor heterogeneity as a consequence of alternative splicing of the intron-rich PTH receptor gene (23, 31) is an attractive hypothesis for explanation of the observed heterogeneous PTH responses in the chondrocyte. However, direct proof for the existence of such receptor isoforms in cartilage is lacking. A more ligand selective isoform of the PTH/PTHrP receptor has been identified and characterized for its unresponsiveness to PTH-related peptide (PTHrP), but this PTH 2 receptor seems to be particularly abundant in pancreas and brain and also recognizes the amino-terminal fragment of PTH (54). However, more recently a novel PTH receptor with specificity for the carboxyl-terminal region of PTH has been characterized in rat osteosarcoma and parathyroid cell lines (20). Moreover, in osteosarcoma cell lines (ROS 17/2.8), this COOH-terminal receptor seemed to be upregulated in response to PTH stimuli (PTH 1-34) that are mediated via the common PTH/PTHrP receptor, implying its role in C-receptor expression (20).

Thus, the possibility remains that one of the above mentioned receptors may be involved in the stimulatory effect of the COOH-terminal part of PTH. Receptor isoforms could also explain the differential effects of central vs COOH-terminal PTH peptides on the induction of Ca^{2+} signaling in distinct cell subsets. Alternatively, Civitelli et al. (11) suggested a nonuniform distribution of functional receptors over the cell surface to explain similar heterogeneous calcium responses to PTH in the osteogenic sarcoma cell line UMR 106. A conformational change in a common receptor molecule could also account for the selective action of the different functional domains of PTH on distinct

subpopulations of chondrocytes. Irrespective of the receptor molecule involved, our results suggest that two distinct functional domains on the PTH molecule can exert different hormonal effects on collagen II and X metabolism by epiphyseal chondrocytes, depending on the differentiation stage of the cells.

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References

- Abou-Samra, A.B., H. Jüppner, T. Force, M.W. Freeman, X.F. Kong, E. Schipani, P. Urena, J. Richards, J.V. Bonventre, J.T. Potts, Jr., et al. 1992. Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intra-cellular accumulation of both cAMP and inositol triphosphates and increases intracellular free calcium. *Proc. Natl. Acad. Sci. USA*. 89:2732-2736.
- Aigner, T., W. Bertling, H. Stöss, G. Weseloh, and K. von der Mark. 1993. Independent expression of fibril-forming collagens I, II, and III in chondrocytes of human osteoarthritic cartilage. *J. Clin. Invest.* 91:829-837.
- Akisaka, T., H. Kawaguchi, G.P. Subita, Y. Shigenaga, and C.V. Gay. 1988. Ultrastructure of matrix vesicles in chick growth plate as revealed by quick freezing and freeze substitution. *Calcif. Tissue Int.* 42:383-393.
- Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1987. *Current Protocols in Molecular Biology*. Greene Publishing Association and Wiley Interscience, New York. 4.9.1-4.9.7.
- Barling, P.M., and N.J. Bibby. 1985. Study of the localization of [^3H]bovine parathyroid hormone in bone by light microscope autoradiography. *Calcif. Tissue Int.* 37:441-446.
- Benay, P.D., and J.D. Schaffer. 1982. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell*. 30:215-224.
- Bringhurst, F.R., G.V. Segre, G.W. Lampman, and J.T. Potts, Jr. 1982. Metabolism of parathyroid hormone by Kupffer cells: analysis by reverse-phase high-performance liquid chromatography. *Biochemistry*. 21:4252-4258.
- Capehart, A.A., and D.M. Biddulph. 1991. Development of PTH-responsive adenylate cyclase activity during chondrogenesis in cultured mesenchyme from chick limb buds. *Calcif. Tissue Int.* 48:400-406.
- Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294-5299.
- Choi, Y.C., G.M. Morris, F.S. Lee, and L. Sokoloff. 1980. The effect of serum on monolayer cell culture of mammalian articular chondrocytes. *Connect Tissue Res.* 7:105-112.
- Civitelli, R., A. Fujimori, S.M. Bernier, P.M. Warlow, D. Goltzman, K.A. Hruska, and L.V. Avioli. 1992. Heterogeneous intracellular free calcium responses to parathyroid hormone correlate with morphology and receptor distribution in osteogenic sarcoma cells. *Endocrinology*. 130:2392-2400.
- Connor, J.A. 1986. Digital imaging of free calcium changes and of spatial gradients in growing processes in single, mammalian central nervous system cells. *Proc. Natl. Acad. Sci. USA*. 83:6179-6183.
- Crabb, I.D., R.J. O'Keefe, J.E. Puzas, and R.N. Rosier. 1992. Differential effects of parathyroid hormone on chick growth plate and articular chondrocytes. *Calcif. Tissue Int.* 50:61-66.
- Erba, H.P., P. Gunning, and L. Kedes. 1986. Nucleotide sequence of the human gamma cytoskeletal actin mRNA: anomalous evolution of non-muscle actin genes. *Nucleic Acids Res.* 14:5275-5294.
- Goltzman, D., A. Peytremann, E. Callahan, G.W. Tregear, and J.T. Potts, Jr. 1975. Analysis of the requirements for parathyroid hormone action in renal membranes with the use of inhibiting analogues. *J. Biol. Chem.* 250:3199-3203.
- Grant, W.T., M.D. Sussman, and G. Balian. 1985. A disulfide-bonded short chain collagen synthesized by degenerative and calcifying zones of bovine growth plate cartilage. *J. Biol. Chem.* 260:3798-3803.
- Grummt, I., C. Soellner, and I. Scholz. 1979. Characterization of a cloned ribosomal fragment from mouse which contains the 18S coding region and adjacent spacer sequences. *Nucleic Acids Res.* 6:1351-1369.
- Gryniewicz, G., M. Poenie, and R.Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440-3450.
- Hale, J.E., and R.E. Wuthier. 1987. The mechanism of matrix vesicle formation. Studies on the composition of chondrocyte microvilli and on the effects of microfilament-perturbing agents on cellular vesiculation. *J. Biol. Chem.* 262:1916-1925.
- Inomata, N., M. Akiyama, N. Kubota, and H. Jüppner. 1995. Characterization of a novel parathyroid hormone (PTH) receptor with specificity for the carboxyl-terminal region of PTH-(1-84). *Endocrinology* 136:4732-4740.
- Iwamoto, M., A. Jikko, H. Murakami, A. Shimazu, K. Nakashima, M. Iwamoto, M. Takigawa, H. Baba, F. Suzuki, and Y. Kato. 1994. Changes in parathyroid hormone receptors during chondrocyte cytodifferentiation. *J. Biol. Chem.* 269:17245-17251.
- Iwamoto, M., A. Shimazu, and M. Pacifici. 1995. Regulation of chondrocyte maturation by fibroblast growth factor-2 and para-thyroid hormone. *J. Orthop. Res.* 13:838-845.
- Jouishomme, H., J.F. Whitfield, B. Chakravarthy, J.P. Durkin, L. Gagnon, R.J. Isaacs, S. MacLean, W. Neugebauer, G. Willick, and R.H. Rixon. 1992. The protein kinase-C activation domain of the parathyroid hormone. *Endocrinology*. 130:53-60.
- Jüppner, H., A.B. Abou-Samra, M. Freeman, X.F. Kong, E. Schipani, J. Richards, L.F. Kolakowski, Jr., J. Hock, J.T. Potts, Jr., H.M. Kronenberg, et al. 1991. A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science (Wash. DC)*. 254:1024-1026.
- Kaji, H., T. Sugimoto, M. Kanatani, A. Miyauchi, T. Kimura, S. Sakakibara, M. Fukase, and K. Chihara. 1994. Carboxyl-terminal PTH fragments stimulate osteoclast-like cell formation and osteoclastic activity. *Endocrinology*. 134:1897-1904.
- Kato, Y., A. Shimazu, K. Nakashima, F. Suzuki, A. Jikko, and M. Iwamoto. 1990. Effects of parathyroid hormone and calcitonin on alkaline phosphatase activity and matrix calcification in rabbit growth-plate chondrocyte cultures. *Endocrinology*. 127:114-118.
- Kirsch, T., and K. von der Mark. 1991. Ca^{2+} binding properties of type X collagen. *FEBS Lett.* 294:149-152.
- Kirsch, T., and M. Pfäffle. 1992. Selective binding of anchoring CII (annexin V) to type II and X collagen and to chondrocalcin (C-propeptide of type II collagen). Implications for anchoring function between matrix vesicles and matrix proteins. *FEBS Lett.* 310:143-147.
- Kirsch, T., and R.E. Wuthier. 1994. Stimulation of calcification of growth plate cartilage matrix vesicles by binding to type II and X collagens. *J. Biol. Chem.* 269:11462-11469.
- Koike, T., M. Iwamoto, A. Shimazu, K. Nakashima, F. Suzuki, and Y. Kato. 1990. Potent mitogenic effects of parathyroid hormone (PTH) on embryonic chick and rabbit chondrocytes. Differential effects of age on growth, proteoglycan, and cyclic AMP responses of chondrocytes to PTH. *J. Clin. Invest.* 85:626-631.
- Kong, X.F., E. Schipani, B. Lanske, H. Joun, M. Karperien, L.H.K. Defize, H. Jüppner, J.T. Potts, Jr., G.V. Segre, H.M. Kronenberg, et al. 1994. The rat, mouse and human genes encoding the receptor for parathyroid hormone and parathyroid hormone-related peptide are highly homologous. *Biochim. Biophys. Res. Commun.* 200:1290-1299.
- Lee, K., J.D. Deeds, A.T. Bond, H. Jüppner, A.B. Abou-Samra, and G.V. Segre. 1993. In situ localization of PTH/PTHrP receptor mRNA in the bone of fetal and young rats. *Bone*. 14:341-345.
- Lewinson, D., and M. Silbermann. 1986. Parathyroid hormone stimulates proliferation of chondroprogenitor cells in vitro. *Calcif. Tissue Int.* 38:155-162.
- Lewinson, D., R. Shurtz-Swirski, P. Shenzer, E. Wingender, H. Mayer, and M. Silbermann. 1992. Structural changes in condylar cartilage following prolonged exposure to the human parathyroid hormone fragment (hPTH) 1-34 in vitro. *Cell. Tissue Res.* 268:257-266.
- Lim, S.K., T.J. Gardella, H. Baba, S.R. Nussbaum, and H.M. Kronenberg. 1992. The carboxy-terminus of parathyroid hormone is essential for hormone processing and secretion. *Endocrinology*. 131:2325-2330.
- Loveys, L.S., D. Gelb, S.R. Hurwitz, J.E. Puzas, and R.N. Rosier. 1993. Effects of parathyroid hormone-related peptide on chick growth plate chondrocytes. *J. Orthop. Res.* 11:884-891.
- Müller, W., and J.A. Connor. 1991. Cholinergic input uncouples Ca^{2+} changes from K-conductance activation and amplifies intradendritic Ca^{2+} changes in hippocampal neurons. *Neuron*. 6:901-905.
- Müller, W., and J.A. Connor. 1991. Dendritic spines as individual neuronal components for synaptic Ca^{2+} responses. *Nature (Lond.)*. 354:73-76.
- Murray, T.M., L.G. Rao, S.A. Muzaffar, and H. Ly. 1989. Human parathyroid hormone carboxyterminal peptide (53-84) stimulates alkaline phosphatase activity in dexamethasone-treated rat osteosarcoma cells in vitro. *Endocrinology*. 124:1097-1099.
- Murray, T.M., M. Sutherland, S.A. Muzaffar, J. Wylie, M. Hausler, and L.G. Rao. 1992. Gene regulation in osteoblastic cells by the carboxyterminal (53-84) fragment of parathyroid hormone: effects of hPTH (53-84) in human osteoblastic SaOS-2 cells. In *Calcium Regulation and Bone Metabolism: Basic and Clinical Aspects*. Vol II. Cohn D.V., C. Gennari and A.H. Tashjian, Jr., editors Excerpta Medica, Amsterdam. pp. 105-109.

41. Pines, M., I. Granot, and S. Hurwitz. 1990. Cyclic AMP-dependent inhibition of collagen synthesis in avian epiphyseal cartilage cells: effect of chicken and human parathyroid hormone and parathyroid hormone-related peptide. *Bone Miner.* 9:23-33.
42. Potts, J.T. Jr., H.M. Kronenberg, and M. Rosenblatt. 1982. Parathyroid hormone: chemistry, biosynthesis, and mode of action. *Adv. Protein Chem.* 35:323-396.
43. Potts, J.T. Jr., G.W. Tregear, H.T. Keutman, H.D. Niall, R. Sauer, L.J. Deftos, B.F. Dawson, M.L. Hogan, and G.D. Aurbach. 1971. Synthesis of a biologically active N-terminal tetratriacontapeptide of parathyroid hormone. *Proc. Natl. Acad. Sci. USA.* 68:63-67.
44. Rao, L.G., and T.M. Murray. 1985. Binding of intact parathyroid hormone to rat osteosarcoma cells: major contribution of binding sites for the carboxyl-terminal region of the hormone. *Endocrinology.* 117:1632-1638.
45. Reichenberger, E., T. Aigner, K. von der Mark, H. Stöss, and W. Bertling. 1991. In situ hybridization studies on the expression of type X collagen in fetal human cartilage. *Dev. Biol.* 148:562-572.
46. Rosenblatt, M., G.V. Segre, G.W. Tregear, G.L. Shepard, G.A. Tyler, and J.T. Potts, Jr. 1978. Human parathyroid hormone: synthesis and chemical, biological, and immunological evaluation of the carboxyl-terminal region. *Endocrinology.* 103:978-984.
47. Schipani, E., H. Karga, A.C. Karaplis, J.T. Potts, Jr., H.M. Kronenberg, G.V. Segre, A.B. Abou-Samra, and H. Jüppner. 1993. Identical complementary deoxyribonucleic acids encode a human renal and bone parathyroid hormone (PTH)/PTH-related peptide receptor. *Endocrinology.* 132:2157-2165.
48. Schlüter, K.D., H. Hellstern, E. Wingender, and H. Mayer. 1989. The central part of parathyroid hormone stimulates thymidine incorporation of chondrocytes. *J. Biol. Chem.* 264:11087-11092.
49. Schmid, I.M., and T.F. Linsenmayer. 1985. Immunohisto-chemical localization of short chain cartilage collagen (type X) in avian tissues. *J. Cell Biol.* 100:598-605.
50. Silberberg, M., and R. Silberberg. 1943. The effects of parathyroid hormone and calcium gluconate on the skeletal tissues of mice. *Am. J. Pathol.* 19:839-859.
51. Silbermann, M., R. Shurtz-Swirski, D. Lewinson, P. Shenzer, and H. Mayer. 1991. In vitro response of neonatal condylar cartilage to simultaneous exposure to the parathyroid hormone fragments 1-34, 28-48, and 53-84 hPTH. *Calcif. Tissue Int.* 48:260-266.
52. Tregear, G.W., J. Van Rietschoten, E. Greene, H.Y. Keutmann, H.D. Niall, B. Reit, J.A. Parsons, and J.T. Potts, Jr. 1973. Bovine parathyroid hormone: minimum chain length of synthetic peptide required for biological activity. *Endocrinology.* 93:1349-1353.
53. Tschan, T., I. Hörler, Y. Houze, K.H. Winterhalter, C. Richter, and P. Bruckner. 1990. Resting chondrocytes in culture survive without growth factors, but are sensitive to toxic oxygen metabolites. *J. Cell Biol.* 111:257-260.
54. Usdin, T.B., C. Gruber, and T.I. Bonner. 1995. Identification and functional expression of a receptor selectively recognizing parathyroid hormone, the PTH2 receptor. *J. Biol. Chem.* 270:15455-15458.
55. von der Mark, K. 1986. Differentiation, modulation and dedifferentiation of chondrocytes. In *Connective Tissue: Biological and Clinical Aspects*. K. Kühn, and T. Krieg, editors. Rheumatology. Vol. 10. Karger, Basel. pp. 272-315.
56. von der Mark, K., S. Frischholz, T. Aigner, F. Beier, J. Belke, S. Erdmann, and H. Burkhardt. 1995. Upregulation of type X collagen expression in osteoarthritic cartilage. *Acta Orthop. Scand. (Suppl.)* (266) 66:125-129.
57. Yamaguchi, D.T., T.J. Hahn, A. Iida-Klein, C.R. Kleeman, and S. Muallem. 1987. Parathyroid hormone-activated calcium channels in an osteoblast-like clonal osteosarcoma cell line. cAMP-dependent and cAMP-independent calcium channels. *J. Biol. Chem.* 262:7711-7718.