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Parathyroid Hormone Inhibits Collagen Synthesis and the Activity of Rat Col1a1 Transgenes Mainly by a cAMP-Mediated Pathway in Mouse Calvariae

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Abstract We examined the effect of parathyroid hormone and various signaling molecules on collagen synthesis and chloramphenicol acetyltransferase activity in cultured transgenic mouse calvariae carrying fusion genes of the rat Col1a1 promoter and the chloramphenicol acetyltransferase reporter. After 48 h of culture, parathyroid hormone, forskolin, dibutyryl cAMP, 8-bromo cAMP, and phorbol myristate acetate inhibited transgene activity, while the calcium ionophore ionomycin had no effect. Pretreatment of calvariae with the phosphodiesterase inhibitor isobutylmethylxanthine potentiated the inhibitory effect of 1 nM parathyroid hormone on transgene activity and collagen synthesis. Parathyroid hormone further inhibited transgene activity and collagen synthesis in the presence of phorbol myristate acetate. Parathyroid hormone inhibition of transgene activity and collagen synthesis was not affected by indomethacin or interleukin-6. After 48 h of culture, parathyroid hormone inhibited chloramphenicol acetyltransferase activity by 50–85% in cultured calvariae carrying transgenes having progressive 5′ upstream deletions of promoter DNA down to −1683 bp. These data show that the inhibitory effect of parathyroid hormone on Col1a1 expression in mouse calvariae is mediated mainly by the cAMP signaling pathway. Prostaglandins and IL-6 are not local mediators of the parathyroid hormone response in this model. Finally, regions of the Col1a1 promoter downstream of −1683 bp are sufficient for parathyroid hormone inhibition of the Col1a1 promoter. J. Cell. Biochem. 77:149–158, 2000.

Key words: type I collagen; Col1a1; osteoblast; bone; transgenic

Parathyroid hormone (PTH) inhibits bone formation and collagen synthesis in a variety of in vitro models. Continuous treatment of cultured rodent calvariae and osteoblastic cells with PTH inhibits type I collagen expression [Bringhurst and Potts, 1982; Dietrich et al., 1976; Kream et al., 1980, 1986; Partridge et al., 1989] and decreases the formation of bone nodules in long-term cultures of rat osteoblastic cells [Bellows et al., 1990]. The biological effects of PTH in bone, kidney, and other target tissues result from its binding to 7-transmembrane PTH/PTHrP receptors [Juppner et al., 1991]. These receptors are coupled to G-proteins, which engage multiple signaling pathway effectors such as adenylate cyclase and phosphoplipase C [Abou-Samra et al., 1992]. PTH treatment of

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bone cells increases the accumulation of the second messengers cAMP [Chase et al., 1969] and diacylglycerol [Reid et al., 1988], which activate the protein kinase A (PKA) and protein kinase C (PKC) signaling pathways, respectively, as well as inositol 1,4,5-trisphosphate [Cosman et al., 1989], which leads to an increase in intracellular free calcium [Reid et al., 1988]. Recently, it has been suggested that PTH may stimulate non-phospholipase C-mediated activation of the PKC pathway [Takasu et al., 1999].

A goal of our work is to elucidate mechanisms by which hormones regulate Col1a1 expression in bone. To map elements that govern the constitutive and hormone-regulated expression of Col1a1 in vivo, we produced transgenic mice that carry fusion genes of Col1a1 promoter DNA linked to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. We previously showed that DNA sequences between −2.3 and −1.7 kb are required for Col1a1 promoter expression in calvariae [Bogdanovic et al., 1994; Krebsbach et al., 1993]. PTH inhibits the activity of ColCAT3.6 and ColCAT2.3, endogenous Col1a1, and transgene mRNA levels and rates of collagen synthesis in cultured transgenic mouse calvariae [Kream et al., 1993]. Forskolin also inhibits CAT activity in transgenic calvariae, suggesting that the cAMP-PKA signaling pathway is affected by PTH. However, activation of the PKC pathway can also decrease collagen synthesis in fetal rat calvariae [Feyen et al., 1988] and Col1a1 transcription rates in MC3T3-E1 cells [Harrison et al., 1990]. It is possible that, in some instances, PTH modulation of gene expression via these pathways may be mediated by the production of local factors that act as either autocrine or paracrine regulators, or both. Two such candidate mediators are prostaglandin E_2 (PGE_2) and interleukin-6 (IL-6). PTH increases PGE_2 production in rodent calvariae [Klein-Nulend et al., 1991] and IL-6 production in neonatal mouse calvariae [Feyen et al., 1989].

PGE_2 decreases collagen synthesis in rodent calvariae [Raisz and Fall, 1990] and Col1a1 gene transcription in rat osteoblastic Py-1a cells [Raisz et al., 1993], while IL-6 has a weak inhibitory effect on collagen synthesis in MC3T3-E1 cells [Fang and Hahn, 1991]. The goals of the present study were to define the signaling pathway by which PTH inhibits type I collagen expression, to determine whether IL-6 and PGE_2 are involved in the PTH response and to map a PTH-responsive region of the Col1a1 promoter, using organ cultures of neonatal transgenic mouse calvariae.

MATERIALS AND METHODS

Materials

Sources for the materials used in this study were as follows: BGJ_b medium from Gibco (Grand Island, NY); bovine PTH(1–34), forskolin, dibutyryl cAMP, 8-bromo cAMP, phorbol myristate acetate (PMA), and indomethacin from Sigma Chemical Co. (St. Louis, MO); ionomycin from LC Laboratories (Woburn, MA); recombinant murine IL-6 from BioSource (Camarillo, CA); oligonucleotides from Pharmacia (Piscataway, NJ); [5-^{3}H]proline (5–10 Ci/mmol) from Amersham (Arlington Heights, IL); [^{3}H]acetyl coenzyme A (200 mCi/mmol), [^{32}P]dCTP (3,000 Ci/mmol), and [^{32}P]dGTP (3000 Ci/mmol) from New England Nuclear Research Products/DuPont (Wilmington, DE); bacterial collagenase from Cooper Biomedical (Malvern, PA) or Sigma; and tissue culture plates from Costar (Cambridge, MA). CD-1 female mice were purchased from the Charles River Breeding Company (Boston, MA).

Transgenic Mouse Lines

The construction and characterization of the ColCAT 3.6 and ColCAT 2.3 fusion genes have been described previously [Lichtler et al., 1989]. The parental construct ColCAT 3.6 contains 3,518 bp of 5′ upstream Col1a1 DNA, 116 bp of untranslated Col1a1 coding DNA, and the CAT reporter gene. Additional deletion constructs were produced using restriction enzyme sites, Bal31 digestion, or synthetic oligonucleotides [Bedalov et al., 1995]. Transgenic mice were produced by the DNA microinjection method of Hogan et al. [1986]. Transgenic founder mice were identified by dot-blot hybridization of DNA to identify the CAT gene. Positive animals in heterozygous litters were identified by subjecting tail extracts to CAT analysis as described below. Matings of founder animals or subsequent heterozygous or homozygous breeders were carried out with ICR mice.

Culture of Mouse Calvariae and Assay of Collagen and Noncollagen Protein Synthesis

Half calvariae were dissected from 6- to 8-day-old neonatal mice and cultured individually in

35-mm plastic tissue culture wells containing 2 ml BGJ_b medium with 1 mg/ml bovine serum albumin (BSA), 100 μg/ml ascorbic acid, and 1 mM proline, as previously described [Kream et al., 1993]. PTH peptides were prepared as a 0.1 mM stock solutions in 1 mg/ml BSA containing 0.001 N HCl and diluted in culture medium at least 1,000-fold. Drugs were added at a 1:1,000 dilution to test cultures, while control cultures received an equivalent volume of vehicle. Calvariae were transferred to fresh medium daily and radiolabeled with [^{3}H]proline (5 μCi/ml) for the final 2 h of the culture period. Each calvaria was extracted in trichloroacetic acid, acetone, and ether, then dried, weighed and homogenized in 1 ml 0.5 M acetic acid. To quantitate the incorporation of [^{3}H]proline into collagenase-digestible protein (CDP) and noncollagen protein (NCP), and to determine percentage collagen synthesized (PCS), calvarial homogenates were digested with purified bacterial collagenase [Diegelmann and Peterkofsky, 1972; Peterkofsky and Diegelmann, 1971].

Measurement of CAT Activity

CAT activity in soluble extracts of calvariae was assayed as described previously [Kream et al., 1993]. Briefly, calvariae were rinsed in phosphate-buffered saline (PBS), pH 7.4, blotted and put individually into a tube with 0.2 ml of a buffer containing 0.25 M Tris-HCl, pH 7.8 and 0.5% Triton X-100. Calvariae were subjected to three cycles of freezing on dry ice, each followed by thawing at 37°C. The extracts were heated at 65°C for 15 min and centrifuged at 14,000*g* for 1 min. A modification of the fluor diffusion assay was used to measure CAT activity in the extracts [Neumann et al., 1987]. A sample of extract was combined with 200 μl of a reaction mix containing 1 mM chloramphenicol, 0.2 μCi [^{3}H]acetyl coenzyme A, and 0.025 M Tris-HCl, pH 7.8 in a 7-ml scintillation vial. The aqueous reaction mix was overlaid with 5 ml of toluene-based scintillation fluid. The vials were incubated at 37°C and counted every hour and CAT activity calculated as previously described [Kream et al., 1993]. CAT activity in each sample was normalized to protein in the extract using the indicator bicinchoninic acid [Smith et al., 1985].

Statistics

Results were analyzed using Student's *t*-test for a two group experiment or one-way analysis of variance (ANOVA) for a multiple group experiment. When using ANOVA, comparisons among groups were made using the Student-Newman-Kuel posthoc test.

RESULTS

Previously, we showed that PTH inhibits the activity of ColCAT3.6 and ColCAT2.3 in cultured transgenic mouse calvariae, suggesting that a PTH-responsive region of the Col1a1 gene is downstream of −2.3 kb [Kream et al., 1993]. To map a PTH-responsive region in the Col1a1 promoter, we examined the effect of PTH on the activity of ColCAT constructs having progressively truncated 5′ promoter fragments (Fig. 1). These data were compared with the effects of PTH on ColCAT3.6 and ColCAT2.3, which have been previously characterized [Kream et al., 1993]. PTH at 10 nM decreased the activity of each transgene by about 50–85% in 48-h cultures of calvariae (Table I). Differences in the magnitude of inhibition could be due to the integration site of the transgene in the different lines or experimental variation. These data indicate that the rat Col1a1 promoter truncated to −1683 bp is still responsive to PTH.

To dissect the signal transduction pathway(s) by which PTH inhibits type I collagen expression, calvariae were cultured with pharmacological agents that activate the PKA, PKC, and calcium signaling pathways. Forskolin at 10 μM inhibited CAT activity, while the calcium ionophore ionomycin at 1 μM was not effective

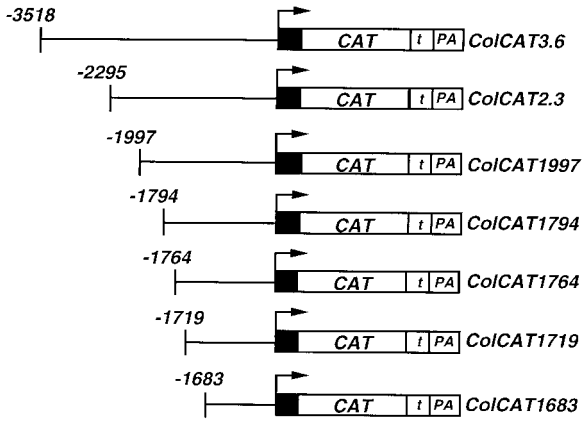

Fig. 1. ColCAT deletion constructs used for the generation of transgenic mice. Each transgene contains a segment of the 5′ flanking region of the Col1a1 gene (straight line), 116 bp of untranslated Col1a1 coding DNA (closed box), the CAT reporter gene, and the SV40 small t intron (t) and polyadenylation sequences (PA).

TABLE I. Effect of Bovine Parathyroid Hormone 1–34 (PTH) on CAT Activity in 48-h Cultures of Transgenic Calvariae†

Construct	Line	CAT (cpm/h/μg protein) Control	PTH 10 nM
ColCAT3.6	14	8,045 ± 584	2,430 ± 390*
ColCAT2.3	5	4,232 ± 230	1,647 ± 286*
ColCAT1997	53	3,153 ± 338	1,150 ± 88*
	54	15,327 ± 1,910	5,215 ± 876*
ColCAT1794	61	12,217 ± 1,925	3,832 ± 848*
	63	4,491 ± 895	1,586 ± 496*
ColCAT1764	286	18,508 ± 1,452	6,950 ± 311*
ColCAT1719	311	4,035 ± 285	1,743 ± 200*
	312	3,115 ± 506	1,108 ± 181*
ColCAT1683	68	1,006 ± 128	492 ± 71*

†Calvariae were cultured for 48 h in the presence or absence of 10 nM PTH. Extracts were prepared from individual calvariae and assayed for CAT activity as described under Materials and Methods. Each value is the mean ±SEM for CAT activity in 5–24 calvariae.
*Significant effect of PTH, $P < 0.05$.

TABLE II. Effect of PTH, PMA, Forskolin, and Ionomycin on CAT Activity in Cultured Transgenic Calvariae†

Construct	Treatment	CAT (cpm/h/μg protein)
ColCAT3.6 (line 2)	Control	8,932 ± 761
	PTH 10 nM	4,571 ± 325*
	PMA 100 nM	5,026 ± 400*
	PMA 10 nM	5,595 ± 435*
	PMA 1 nM	8,885 ± 302
ColCAT3.6 (line 14)	Control	7,580 ± 691
	PTH 10 nM	2,759 ± 176*
	Ionomycin 1 μM	5,684 ± 1193
	PMA 100 nM	6,320 ± 587
	Forskolin 10 μM	3,066 ± 180*

†Calvariae were cultured for 48 h in the presence or absence of effectors. Each value is the mean ±SEM of 4–6 samples.
*Different from control, $P < 0.05$.

(Table II). PMA at 10 and 100 nM was also inhibitory (Table II). Because the extent of the inhibitory effect of 100 nM PMA was variable among individual experiments as shown in Table II, the results from nine experiments were pooled. These data showed that the inhibitory effects of PMA and PTH on ColCAT3.6 activity were equivalent (Table III).

To further test the involvement of the PKC pathway in the PTH response, calvariae were treated with a maximal dose of PMA (100 nM)

TABLE III. Comparison of the Effects of PTH and PMA on CAT Activity in Cultured Transgenic Calvariae†

Construct	Treatment	CAT (% of control)
ColCAT3.6	Control	100 ± 3
	PTH 10 nM	57 ± 3*
	PMA 100 nM	64 ± 3*

†Calvariae were cultured with effectors for 48 h. CAT activity is expressed relative to the control value, set at 100. Each value is the mean ±SEM of 40–48 samples with ColCAT3.6 lines 2, 14, and 18.
*Different from control, $P < 0.05$.

for 24 h and then treated with 10 nM PTH for an additional 24 h. The effects of these agents on the labeling of collagenase-digestible protein (CDP), the labeling of noncollagen protein (NCP), percentage collagen synthesis and CAT activity were measured (Table IV). PMA alone inhibited CAT activity, CDP labeling and percentage collagen synthesis. PTH decreased CAT activity to the same extent as PMA but had a greater inhibitory effect than PMA on CDP labeling and percentage collagen synthesis. PTH maintained its inhibitory effects on CAT activity, CDP labeling and percentage collagen synthesis in the presence PMA. These data suggest that PTH and PMA use separate pathways for their inhibitory effects on collagen synthesis and rat Col1a1 promoter activity.

Previously, we showed that PTH at a dose range of 1–0 nM inhibits ColCAT3.6 and ColCAT2.3 activity. If the cAMP pathway is involved in the PTH response, the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) should enhance the inhibitory activity of a submaximal PTH dose. PTH at 1 nM inhibited CDP labeling and the percentage collagen synthesis but did not inhibit CAT activity (Table V). In the presence of 0.1 mM IBMX, 1 nM PTH had an even greater inhibitory effect on CDP labeling and percentage collagen synthesis and inhibited CAT activity to the same extent as 10 nM PTH (Table V).

The cAMP analogues, dibutyryl cAMP and 8-bromo cAMP at 1 mM, inhibited CAT activity, CDP labeling and the percentage collagen synthesis (Table V). 8-Bromocyclic AMP also inhibited NCP labeling (Table V). These analogues at 0.1 mM were not inhibitory (data not shown). The cAMP analogues were more effective than 10 nM PTH in inhibiting CDP labeling and percentage collagen synthesis; however, PTH

TABLE IV. Effect of PTH on the Labeling of Collagenase-Digestible Protein (CDP), Labeling of Noncollagen Protein (NCP), Percentage Collagen Synthesis (PCS), and CAT Activity in Calvariae Precultured in the Presence or Absence of PMA†

Treatment	CDP	NCP		CAT
0–24 h/24–48 h	dpm/μg dry weight		PCS	cpm/h/μg protein
Control/control	55.4 ± 2.3	51.3 ± 5.5	17.9 ± 1.4	10,638 ± 795
Control/PTH	19.3 ± 1.9*	52.1 ± 3.8	6.4 ± 0.4*	7,227 ± 625*
PMA/PMA	37.9 ± 3.1*	52.1 ± 3.8	12.3 ± 1.1*	7,432 ± 475*
PMA/PMA + PTH	16.8 ± 0.7**	50.9 ± 2.9	5.8 ± 0.2**	4,405 ± 293**

†Transgenic calvariae carrying ColCAT3.6 were cultured for 24 h in the presence or absence of 100 nM PMA. The bones in each treatment group were then divided and cultured in medium for an additional 24 h in medium as indicated above. At the end of culture, bones were incubated with [^{3}H]proline for 2 h to measure CDP labeling, NCP labeling, and PCS or analyzed directly for CAT activity. Each value is the mean ±SEM of 12–15 samples.
*Different from the control/control group, $P < 0.05$.
**Different from the PMA/PMA group, $P < 0.05$.

TABLE V. Effect of PTH, Dibutyryl cAMP ($(Bu)_2$cAMP), 8-bromo cAMP (8BrcAMP), and Isobutylmethylxanthine (IBMX) on the Labeling of Collagenase-Digestible Protein (CDP), Labeling of Noncollagen Protein (NCP), Percentage Collagen Synthesis (PCS), and CAT Activity in Cultured Mouse Calvariae†

Treatment	CDP	NCP	PCS	CAT (line 2)
	dpm/μg dry weight			cpm/h/μg protein
Control	53.2 ± 2.0	41.4 ± 1.9	19.3 ± 0.5	11,600 ± 540
PTH 10 nM	21.6 ± 2.1*	41.1 ± 5.0	8.8 ± 0.4*	6,174 ± 283*
PTH 1 nM	31.9 ± 2.9*	54.6 ± 3.4	9.7 ± 0.6*	9,435 ± 776
IBMX 0.1 mM	47.0 ± 3.1	47.3 ± 2.5	15.5 ± 0.4*	12,099 ± 878
IBMX + PTH 1 nM	17.2 ± 2.7**,‡	43.2 ± 4.6	6.6 ± 0.4**,‡	5,869 ± 349**,†
$(Bu)_2$cAMP 1 mM	11.8 ± 1.9*	37.3 ± 4.2	5.5 ± 0.6*	5,365 ± 336*
8BrcAMP 1 mM	5.5 ± 1.5*	24.4 ± 4.1*	3.4 ± 0.6*	5,321 ± 375*

†Calvariae were cultured with effectors for 48 h. When IBMX was used, calvariae were precultured for 2 h in the presence or absence of IBMX prior to addition of PTH. For protein labeling data, each value is the mean ±SEM of 5–7 cultures from two experiments. For CAT activity data (ColCAT3.6, line 2 mice), each value is the mean ±SEM of 13–29 samples.
*Different from control, $P < 0.05$.
**Different from IBMX, $P < 0.05$.
‡Different from 1 nM PTH without IBMX, $P < 0.05$.

and the cAMP analogues inhibited CAT activity to a comparable extent. This may indicate that in addition to transcriptional repression, cAMP inhibits type I collagen expression by additional mechanisms. Alternatively, Col1a1 sequences not represented in our constructs may be involved in PTH repression of Col1a1 expression.

As another means of determining the signaling pathways of PTH, ColCAT3.6 calvariae (line 2) were treated with 10 nM PTH(1–34) and PTH(3–34) for 48 h. PTH(3–34) has greatly reduced ability to signal through the cAMP-PKA pathway because it lacks the first two amino acids of PTH(1–34) but can still signal via the PKC pathway [Fujimori et al., 1992]. PTH(3–34) decreased CAT activity to 74 ± 15% the level in vehicle-treated calvariae (n = 5 experiments). This inhibitory effect of PTH(3–34) was not significant and was variable among experiments. In the same experiments, PTH(1–34) decreased CAT activity to 49 ± 12% the level in vehicle-treated calvariae (n = 5 experiments, $P < 0.05$).

PTH treatment of calvariae might result in production of a local factor that could act as an autocrine/paracrine mediator of the PTH inhibitory response. Two such candidate mediators are PGE_2 and IL-6. Therefore, we tested the effects of these agents on collagen synthesis and CAT activity. The addition of 100 or 1,000 nM PGE_2 to mouse calvarial cultures for 48 h had little or no effect on CAT activity in transgenic calvariae [Harrison et al., 1998]. Treatment of cultured calvariae for 48 h with the cyclooxygenase inhibitor indomethacin did not

TABLE VI. Effect of PTH on Labeling of Collagenase-Digestible Protein (CDP), Labeling of Noncollagen Protein (NCP), Percentage Collagen Synthesis (PCS), and CAT Activity in Cultured Calvariae in the Presence or Absence of Indomethacin (Indo)†

	CDP	NCP		CAT (Line 2)	CAT (Line 5)
Treatment	% of control		PCS	% of control	
Control	100.0 ± 5.2	115.5 ± 12.4	14.9 ± 1.1	100.0 ± 5.2	100.0 ± 8.2
PTH 10 nM	53.1 ± 6.1*	150.0 ± 21.3	7.2 ± 0.8*	39.5 ± 2.5*	41.2 ± 3.5*
Indo 1 μM	92.1 ± 12.5	106.3 ± 12.0	14.0 ± 0.9	88.7 ± 6.8	95.9 ± 5.9
Indo + PTH	46.5 ± 4.6**	131.4 ± 17.5	6.9 ± 0.5**	47.7 ± 5.0**	36.1 ± 3.3**

†Calvariae were cultured for 48 h in the presence or absence of PTH and Indo. For the protein labeling data, each value is the mean ±SEM of 17 cultures from four experiments with lines ColCAT3.6 (line 14) and ColCAT2.3 (line 5) mice. For the CAT measurements, each value is the mean ±SEM of 7–15 cultures from two experiments with line 14 mice and three experiments with line 5 mice. CDP labeling, NCP labeling and CAT activity are expressed relative to the control value, set to 100.
*Different from control, $P < 0.05$.
**Different from Indo, $P < 0.05$.

TABLE VII. Effect of PTH and Recombinant Murine IL-6 on Labeling of Collagenase-Digestible Protein (CDP), Labeling of Noncollagen Protein (NCP), Percentage Collagen Synthesis (PCS), and CAT Activity in Cultured Calvariae†

	CDP	NCP		CAT
Treatment	dpm/μg dry weight		PCS	cpm/h/μg protein
Control	34.5 ± 3.9	29.8 ± 2.8	17.7 ± 0.7	10,403 ± 500
IL-6 100 ng/ml	34.4 ± 3.1	32.1 ± 2.2	16.6 ± 1.1	10,084 ± 513
PTH 10 nM	14.7 ± 1.7*	37.9 ± 2.9	6.6 ± 0.5*	4,955 ± 792*

†Calvariae carrying ColCAT3.6 (line 14) were cultured for 48 h in the presence of effectors. Each value is the mean ±SEM of eight samples from two experiments for protein labeling or four samples from a representative experiment for CAT activity. Similar results for CAT activity were found in three additional experiments.
*Different from control, $P < 0.05$.

affect CAT activity, CDP labeling, NCP labeling or percentage collagen synthesis and did not alter the inhibitory effect of 10 nM PTH on these parameters (Table VI). IL-6 at 100 ng/ml had no effect on CAT activity, CDP labeling, NCP labeling, or percentage collagen synthesis in cultured mouse calvariae (Table VII). Taken together, these data suggest that neither PGE_2 nor IL-6 alone mediates the inhibitory effect of PTH on type I collagen expression in mouse calvariae.

DISCUSSION

Organ cultures of calvariae from transgenic mice containing rat Col1a1 promoter fragments linked to the CAT reporter gene were used as a model to study PTH regulation of type I collagen synthesis. Transgenic calvariae provide a unique model for studying the hormonal regulation of type I collagen expression. Tissue structure and potentially important cell-cell and cell-matrix interactions are preserved. Osteoblasts within intact calvariae maintain a high level of type I collagen synthesis and Col1a1 promoter activity compared with cultured osteoblastic cells [Krebsbach et al., 1993].

In the present study, we showed that forskolin, dibutyryl cAMP and 8-bromo cAMP inhibited collagen synthesis and CAT activity in calvariae. We demonstrated that the phosphodiesterase inhibitor IBMX enhanced the inhibitory response of a submaximal dose of PTH. In this mouse calvarial model, PMA also inhibited Col1a1 promoter activity and collagen synthesis. However, PTH had additional inhibitory effects in the presence of PMA and PTH(3–34), which has much reduced ability to signal via the cAMP-PKA pathway, had little effect on Col1a1 promoter activity. Taken together, our findings indicate that PTH inhibits type I collagen synthesis and Col1a1 promoter activity mainly by the cAMP-PKA signaling pathway. This conclusion is in agreement with that of other studies that used organ cultures of rodent calvariae and osteoblastic cells for assessments of collagen synthesis rates [Bringhurst and

Potts, 1982; Dietrich et al., 1976; Kano et al., 1992]. Many effects of PTH on gene expression in bone cells are mediated at least in part via the cAMP-PKA pathway [Partridge et al., 1994], including collagenase [Scott et al., 1992], c-fos [Pearman et al., 1996], insulin-like growth factor I [McCarthy et al., 1990], IL-6 [Greenfield et al., 1995; Huang et al., 1998], and prostaglandin synthase-2 [Tetradis et al., 1996, 1997]. The anabolic effect of PTH on bone formation requires the cAMP-PKA pathway [Armamentovillareal et al., 1997; Goltzman, 1999; Rixon et al., 1994; Whitfield and Morley, 1995]. However, the PKC pathway is involved in some PTH-regulated functions [Lowik et al., 1985] such as PTH-induced osteoclast-like cell formation [Kaji et al., 1993], bone resorption [Herrmann-Erlee et al., 1983] and osteoblastic proliferation [Somjen et al., 1990].

In preliminary experiments, we found that cycloheximide partially blocked the inhibitory effect of PTH on CAT mRNA levels, suggesting that protein synthesis is required for full PTH responsiveness (Z. Bogdanovic and B. Kream, unpublished data). Likewise, the induction of collagenase gene expression by PTH is blocked by cycloheximide indicating a requirement for ongoing protein synthesis [Scott et al., 1992]. It is possible that PTH inhibits Col1a1 promoter activity by increasing the synthesis of a protein that binds to, and directly inhibits, promoter activity. PTH might also decrease the synthesis of a stimulatory *trans*-acting factor or increase the expression of factor that blocks the activity of a stimulatory *trans*-acting factor. Alternatively, the inhibitory effect of PTH might be due to the production of a local autocrine/paracrine mediator. Two potential candidates are PGE_2 and IL-6. PTH induces prostaglandin synthase-2 gene expression and PGE_2 production in rodent calvariae [Kawaguchi et al., 1994; Klein-Nulend et al., 1991; Tetradis et al., 1996, 1997].

PGE_2 decreases Col1a1 gene transcription, collagen synthesis, and ColCAT3.6 activity in rat osteoblastic Py-1a cells [Raisz et al., 1993]. PTH also increases IL-6 mRNA and protein expression in mouse calvariae and osteoblastic cells [Feyen et al., 1989; Greenfield et al., 1995, 1996; Huang et al., 1998; Lowik et al., 1989]. However, the effects of IL-6 on osteoblast function are modest. IL-6 has only a very weak inhibitory effect on collagen synthesis in mouse calvariae and MC3T3-E1 cells [Fang and Hahn, 1991]. IL-6 at high concentrations decreases the formation of bone nodules in mineralizing cultures of rat osteoblastic cells [Hughes and Howells, 1993]. Based on the data presented in this article, we conclude that in transgenic mouse calvarial organ cultures, the inhibitory effect of PTH is not mediated solely by PGE_2 or IL-6.

Promoter mapping experiments indicate that the PTH-responsive region of the Col1a1 promoter is located downstream of −1683 bp. Studies from our laboratories indicate that a 13-bp region of the Col1a1 gene between −1683 and −1670 bp is required for high expression of the promoter in calvariae of transgenic mice [Dodig et al., 1996]. Deletion of the Col1a1 promoter to −1670 bp abolishes activity in calvariae [Bogdanovic et al., 1994]. Therefore, we are currently generating ColCAT constructs that have internal deletions and mutations of regions downstream of −1683 bp to search for PTH-responsive sequences in the rat Col1A1 promoter. Proximal DNA sequences have been shown to mediate basal expression [Karsenty and de Crombrugghe, 1990] and transforming growth factor-β (TGFβ) regulation [Jimenez et al., 1994] of the Col1a1 gene in fibroblasts.

In contrast with its chronic inhibitory activity in vitro, PTH given in a transient manner to organ cultures of bone can stimulate collagen synthesis [Canalis et al., 1989; Linkhart et al., 1988]. It has been suggested that IGF-I may mediate the anabolic effect of PTH on bone formation, at least in vitro [Canalis et al., 1989; Linkhart and Mohan, 1989], although other local factors may be involved as well. Likewise, PTH given intermittently in vivo has an anabolic effect on bone mass [Gunness-Hey and Hock, 1983; Kalu et al., 1970; Tam et al., 1982] while continuous administration of PTH in vivo does not [Hock and Gera, 1992]. The anabolic effect of PTH on bone is preceded by an inhibitory phase [Howard et al., 1980; Johnston and Deiss, 1965]. We reason that in the presence of continuous PTH, the inhibitory effect is sustained while with transient PTH exposure, the inhibitory effect is reversed once PTH is cleared. Transgenic mice that carry Col1a1 transgenes will be excellent models for determining mechanisms by which PTH affects bone formation since these transgenes are highly expressed by

osteoblasts [Pavlin et al., 1992] and are regulated rapidly in response to PTH.

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