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A Carboxyl-Terminal Peptide from the Parathyroid Hormone-Related Protein Inhibits Bone Resorption by Osteoclasts*

ANNA J. FENTON†, BRUCE E. KEMP, G. NEIL KENT, JANE M. MOSELEY, MING-HAO ZHENG, DOROTHY J. ROWE, JOANNE M. BRITTO, T. JOHN MARTIN, AND GEOFFREY C. NICHOLSON

Department of Medicine, University of Western Australia, Fremantle Hospital (A.J.F., M.-H.Z., D.J.R., J.M.B., G.C.N.), Fremantle, Western Australia 6160; St. Vincent's Institute of Medical Research and Department of Medicine, University of Melbourne (B.E.K., J.M.M., T.J.M.), Fitzroy, Victoria 3065; and the Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital (G.N.K.), Nedlands, Western Australia 6009, Australia

ABSTRACT. PTH-related protein (PTHrP) interacts, via its amino-terminal 34 residues, with PTH receptors on osteoblasts to stimulate osteoclastic bone resorption indirectly. We now report that mature hPTHrP-(1–141) (EC_{50}, $\sim10^{-11}$ M) and a carboxyl-terminal fragment, PTHrP-(107–139) (EC_{50}, $\sim10^{-15}$ M), are potent inhibitors of resorption in an isolated rat osteoclast bone resorption assay, whereas hPTHrP-(1–108) and hPTHrP-(1–34) are inactive in this respect. PTHrP-(107–139) also inhibits resorption in a rat long bone organ culture system and reduces osteoclast spreading. PTHrP-(107–139) does not act on osteoclasts via a cAMP signal transduction mechanism, but its effects may be mediated by protein kinase-C. This previously unrecognized action of PTHrP, to inhibit osteoclastic bone resorption directly, indicates that PTHrP may be a precursor of multiple biologically active peptides with differing physiological functions. (*Endocrinology* **129:** 1762–1768, 1991)

THE MATURE PTH-related protein (PTHrP) (1) comprises 139, 141, or 173 amino acid residues, predicted by alternate mRNA splicing (1–3). Homology with PTH is restricted to the amino-terminal portion, with 8 of the first 13 residues being identical. This permits PTHrP to interact with PTH receptors on osteoblasts to stimulate bone resorption and in the kidney to restrict calcium excretion and promote phosphate and cAMP excretion (4–6). Outside this region there is no homology with PTH, although there is very strong interspecies conservation of the PTHrP sequence up to residue 111, suggesting that residues 35–111 may have other functions unrelated to PTH-like activities (7). Indeed, there are many potential proteolytic sites within the PTHrP molecule, and there is evidence for cleavage products in the circulation (8). We have previously identified a unique action of PTHrP on calcium and phosphate transport in the placenta which is not shared with PTHrP-(1–34) or PTH (9, 10), suggesting that biological activities may not be confined to the PTH homologous portion of the molecule.

Whereas the classical resorption-promoting agents (PTH, PTHrP, 1,25-dihydroxyvitamin D_3, and several cytokines) act indirectly on the osteoclast via osteoblasts (6, 11, 12), only a few factors [calcitonin (CT), prostaglandins, macrophage colony-stimulating factor, and glucocorticoids] act directly on the osteoclast (13–15). To differentiate direct from indirect effects of hormones and other agents on osteoclasts, an isolated osteoclast bone resorption assay has been developed. This employs cells disaggregated from neonatal rat long bones and settled onto thin slices of devitalized bovine cortical bone (16). Although osteoblasts and other nonosteoclast cells are present in these cultures, they are widely dispersed, producing functionally pure populations of osteoclasts (11, 14, 17).

Using this assay, together with a bone organ culture system, we have found that the carboxyl-terminal portion of PTHrP, PTHrP-(107–139), is a potent inhibitor of osteoclastic bone resorption.

Materials and Methods

Purified osteoclast cultures

Osteoclasts were isolated from neonatal female Wistar rats by curetting the long bones into culture medium (HEPES-

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Address requests for reprints to: Dr. Anna J. Fenton, Department of Medicine, University of Melbourne, Geelong Hospital, Geelong, Victoria 3220, Australia.
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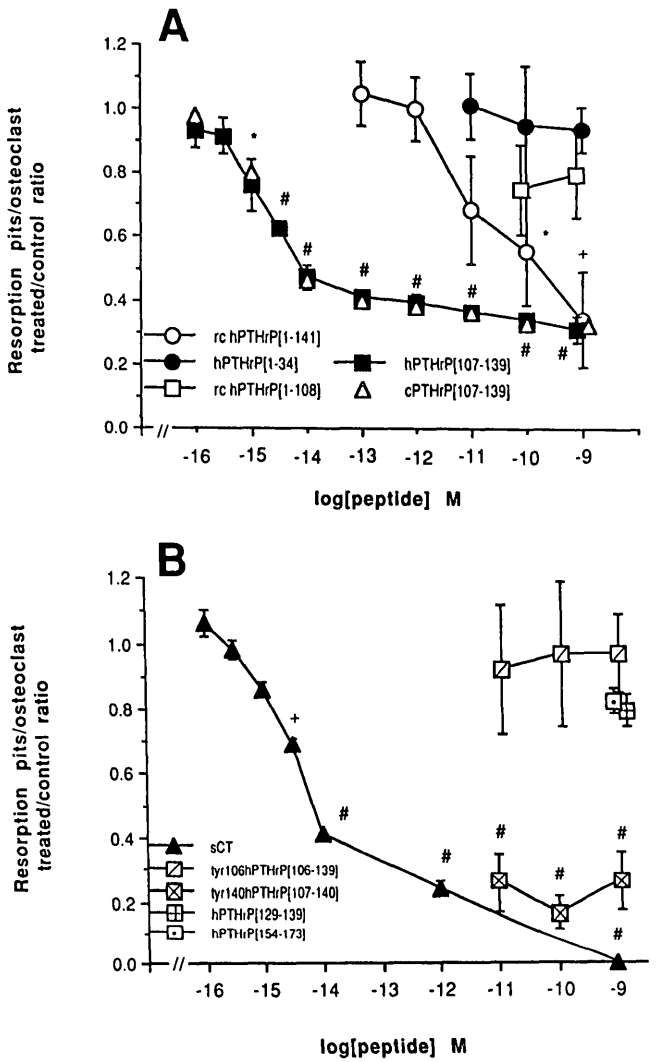

FIG. 1. A and B, Representative experiments demonstrating the effect of PTHrP analogs and sCT on osteoclastic bone resorption by isolated osteoclasts cultured for 24 h on thin slices of bone. The dose-response curves for hPTHrP-(107–139) and chicken PTHrP-(107–139) each represent the results from two pooled experiments. The dose-response curves for the other peptides represent individual experiments. Because of interassay variation in control resorption, the mean for each point has been expressed as the treatment/control ratio (±SEM) to allow direct comparison between experiments. In 14 separate experiments, resorption was maximally inhibited by hPTHrP-(107–139) (10^{-9} M) to 28 ± 3% of the control value. The plan area of the individual resorption pits was not significantly altered by hPTHrP-(107–139) [control, 470 ± 71 μm^2 (n = 333); hPTHrP-(107–139) (10^{-9} M), 447 ± 68 μm^2 (n = 133)]. hPTH(1–34) (10^{-9} M) did not stimulate resorption in these cultures. rc, Recombinant; h, human; c, chicken. ★, $P < 0.05$; +, $P < 0.005$; #, $P < 0.001$. The significance values adjacent to the hPTHrP-(107–139) and cPTHrP-(107–139) points apply to both.

buffered medium 199, Flow, Sydney, Australia). The resulting cell suspension was allowed to settle onto 100-μm thick slices of devitalized bovine cortical bone for 20 min before rinsing. After this brief settling period, the majority of contaminating cells were removed in the rinsing step, resulting in a functionally pure population of osteoclasts (11, 14, 17). The bone slices were then incubated in tissue culture medium [Eagle's Minimum Essential Medium (Flow) supplemented with 100 IU/ml benzylpenicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum] at 37 C in 95% air-5% CO_2, pH 7.3, in the presence or absence of test agents. After 24 h, the cells were fixed and reacted cytochemically for tartrate-resistant acid phosphatase (TRACP) (18), and the number of multinucleated cells with strong reactions for TRACP (*i.e.* osteoclasts) was counted. The cells were then stripped by ultrasonication in 0.25 M NH_4OH before processing for scanning electron microscopy. The entire surface of each bone slice was scanned, and the number of resorption pits was counted (11–17). There was a minimum of six replicates for each concentration point for each peptide. Each experiment was repeated at least three times. Statistical significance was determined using Student's *t* test to compare each treatment with the control.

Coculture experiments

Twenty-four hours before these experiments, osteoblast-like cloned osteogenic sarcoma cells (UMR 106–01) (19) were settled onto the bone slices to achieve 75% confluency at the time of commencement of coculture. Osteoclasts were then settled onto the same bone slices. The remainder of the experiments were performed as described above.

Prolonged osteoclast cultures

Osteoclasts were isolated as described above and then cultured on bone slices for up to 96 h. At 24-h intervals, selected slices were fixed, and the numbers of osteoclasts and resorption pits were counted.

Osteoclast spreading assay

Osteoclasts were isolated, as previously described (20–22), into eight-well chamber slides (TissueTek, Nunc Inc., Melbourne, Australia) and incubated at 37 C in control medium for 1 h. For the time-course experiments, all cells were incubated for a further 7 h. PTHrP-(107–139) (10^{-11} M) was added to the wells at various times (10 min to 7 h) before the termination of the experiments. All cells were then fixed in warm formalin-saline for 30 min, stained with Geimsa, and air dried. The slides were mounted in DePeX, BDH Ltd., Melbourne, Australia and examined under bright-field microscopy. The plan areas of the first 50 multinucleate osteoclasts (>2 nuclei) randomly seen were quantified using an image analysis system (MD-20, Leading Edge Pty. Ltd., Adelaide, Australia), and the number of nuclei counted. For each osteoclast, the measured plan area was divided by the number of nuclei contained within the cytoplasm. For the dose-response experiments, the cells were incubated with vehicle or increasing concentrations of PTHrP-(107–139) (3×10^{-16}-10^{-9} M) for 1 h. The cells were then processed and analyzed, as described above.

Peptide preparation and purification

Recombinant human (h) PTHrP-(1–141) and recombinant hPTHrP-(1–108) were prepared and purified as previously described (23). hPTHrP-(107–139), Thr Arg Ser Ala Trp Leu Asp Ser Gly Val Thr Gly Ser Gly Leu Glu Gly Asp His Leu Ser Asp Thr Ser Thr Thr Ser Leu Glu Leu Asp Ser Arg-COOH, and

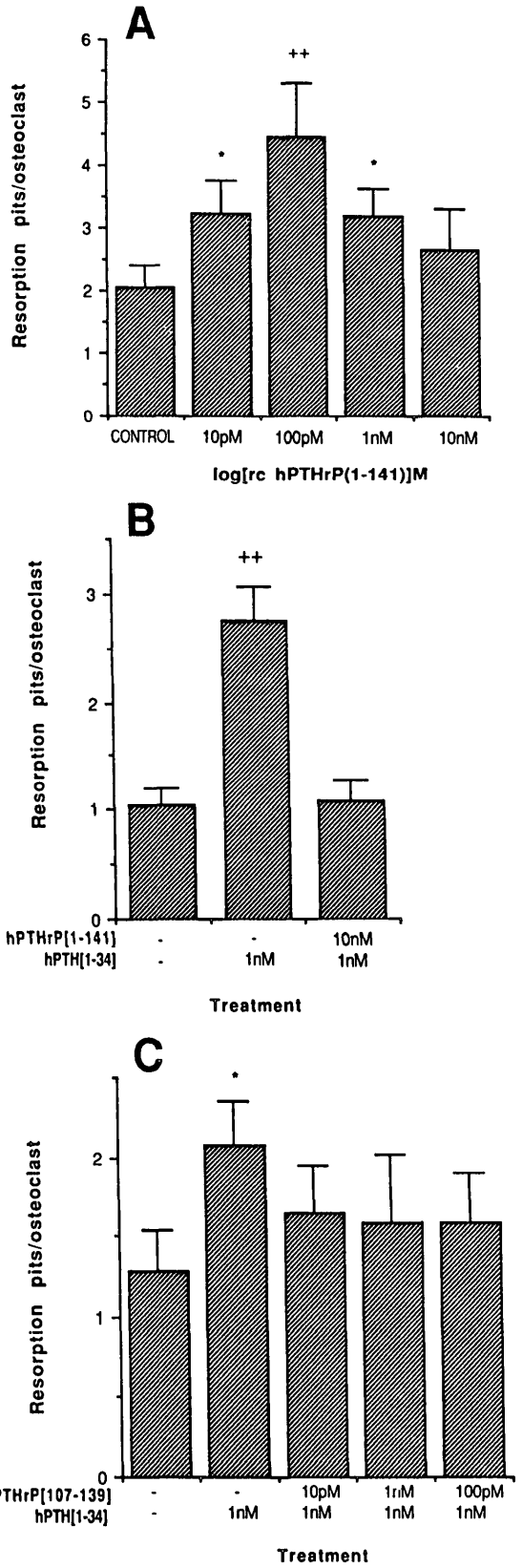

FIG. 2. Effects of PTHrP and PTH on bone resorption in cocultures of rat osteoclasts and the osteoblast-like cell line UMR 106-01. UMR 106-01 cells were settled onto bone slices 24 h before the isolation and culture of osteoclasts. Resorption was assessed as the number of resorption pits formed on each bone slice per multinucleated osteoclast after 24 h of coculture. A, Recombinant hPTHrP-(1-141) (10^{-11}-10^{-8} M) alone. B, hPTH-(1-34) (10^{-9} M) alone or with recombinant hPTHrP-(1-141) (10^{-8} M) C, hPTH-(1-34) (10^{-9} M) alone or with hPTHrP-(107-139) (10^{-11}-10^{-9} M). rc, Recombinant; h, human. ++, $P < 0.02$; ★, $P < 0.05$ (compared to control).

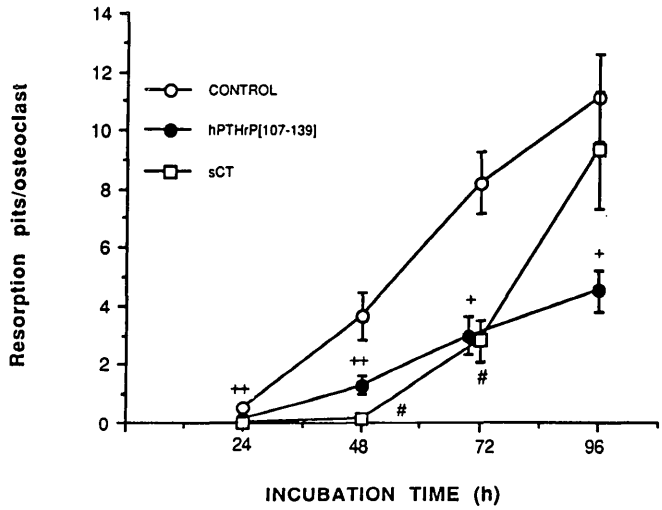

FIG. 3. Effects of PTHrP-(107–139) (10^{-9} M) and sCT (10^{-10} M) on bone resorption in prolonged osteoclast cultures. Osteoclasts were isolated and treated as described, then cultured on bone slices for up to 96 h. At 24-h intervals, selected slices were fixed, and the numbers of osteoclasts and resorption pits were counted. ++, $P < 0.02$; +, $P < 0.005$; # $P < 0.001$.

other peptides were synthesized as carboxyl-terminal amides, using an Applied Biosystems Peptide Synthesizer (model 430, Foster City, CA). After hydrogen fluoride cleavage, peptides were extracted from the resin with 60% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid and lyophilized. The crude peptides were purified by ion exchange and reverse phase chromatography (2.5 × 90-cm column; C 18; 20–30 μm; 300-Å resin; Vydac, Hesperia, CA) with an acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The composition of the synthetic peptides was verified by quantitative amino acid analysis, using a Beckman 6300 amino acid analyzer (Palo Alto, CA).

cAMP assay

Osteoclasts were isolated, as described above, into 12-well tissue culture plates (Linbro, Sydney, Australia). The cultures were pretreated with isobutylmethylxanthine (1 mM) for 20 min before administration of test peptides for 10 min. cAMP was extracted with 95% (vol/vol) ethanol/1 mM HCl and measured by RIA (21). Four to six replicate wells were used in each treatment group.

Organ culture assay

Bone resorption was assessed by the release of previously incorporated ^{45}Ca from fetal rat long bones according to the method of Raisz and Niemann (24). Bones were cultured in modified BGJ medium (Gibco, Grand Island, NY) supplemented with 1% (wt/vol) BSA in the presence or absence of

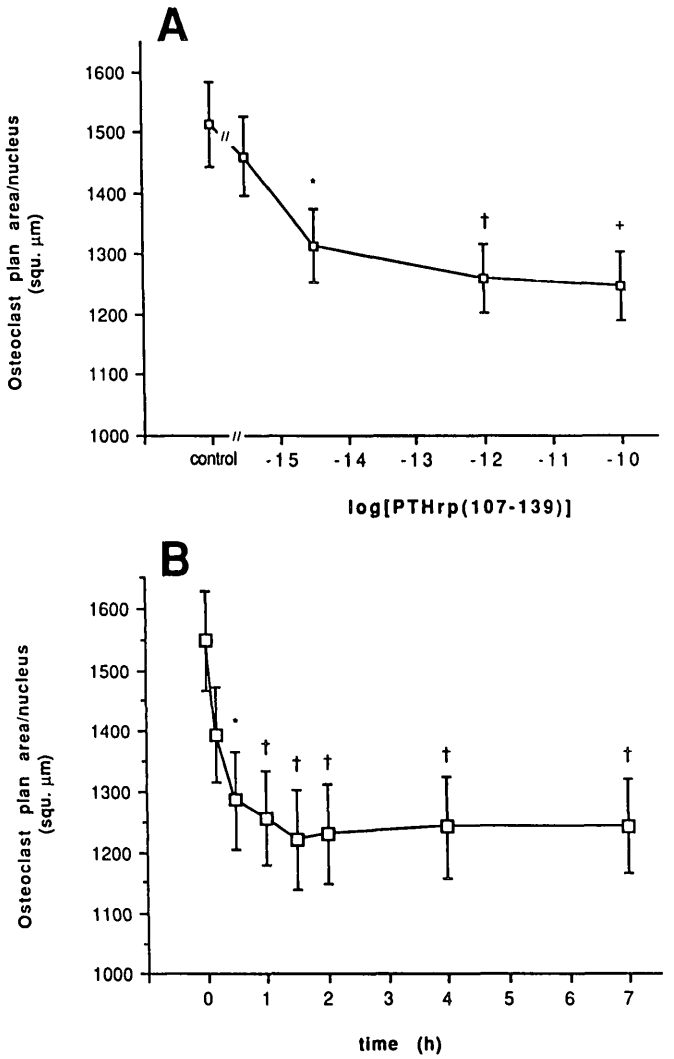

FIG. 4. Effect of PTHrP-(107–139) on osteoclast cytoplasmic spreading. Osteoclasts incubated in glass chamber slides were treated with PTHrP-(107–139) as indicated, fixed in formalin-saline, and stained. The plan area and nuclear number of 50 osteoclasts in each group were recorded, and the ratio osteoclast plan area/nuclear number was calculated for each group. The *points* represent the mean ± SEM. ★, $P < 0.05$; † $P < 0.01$. A, Dose-response. Osteoclasts were treated with increasing concentrations of PTHrP-(107–139) (3×10^{-16}-10^{-9} M) for 1 h. B, Time course. Osteoclasts were treated with PTHrP-(107–139) (10^{-11} M) for the indicated times.

hPTHrP-(107–139) (10^{-8} M) or salmon CT (sCT; 10^{-8} M) for 5 days, with a change of medium at 2 days.

Results

Purified osteoclast cultures

In these experiments we observed that recombinant hPTHrP-(1–141) inhibited resorption at concentrations of 10^{-10} M and above, an effect not shared with synthetic hPTHrP-(1–34) and recombinant hPTHrP-(1–108) (19) (Fig. 1A). This suggested that the inhibitory effect was mediated by the carboxyl-terminal portion of hPTHrP-(1–141). Indeed, we subsequently found that human and chicken PTHrP-(107–139) were potent inhibitors of bone resorption (Fig. 1A). The effect of PTHrP-(107–139) was achieved at concentrations several orders of magnitude less than those of hPTHrP-(1–141) (EC_{50}, $\sim 10^{-15}$ M compared to 10^{-11} M), and dose-response curves were similar to those with sCT (Fig. 1B), which acts directly on osteoclasts (25) to inhibit bone resorption. However, the amplitude of the maximum response to sCT was consistently greater. The importance of the free amino-terminus of hPTHrP-(107–139) was shown by the fact that Tyr^{140}-hPTHrP-(107–140) was active, but Tyr^{106}-hPTHrP-(106–139) was not (Fig. 1B). Other synthetic peptides without any effect on osteoclastic bone resorption were hPTHrP-(129–139), hPTHrP-(129–141), and hPTHrP-(154–173).

TABLE 1. Effect of hPTHrP-(107-139) on bone resorption in organ culture

Treatment	% Release of available ^{45}Ca/5 days		No. of pairs
	Control	Treated	
Exp 1			
hPTHrP-(107-139)	17.9 ± 0.4	12.0 ± 0.5[a]	3
sCT	17.2 ± 0.6	13.1 ± 1.0[a]	3
Exp 2			
hPTHrP-(107-139)	18.4 ± 1.3	14.2 ± 0.8[a]	5
sCT	19.3 ± 3.0	11.1 ± 1.7[a]	6
Exp 3			
hPTHrP-(107-139)	25.6 ± 2.1	20.4 ± 1.3[a]	4
sCT	20.6 ± 1.4	15.8 ± 1.5[a]	5

Values are the mean ± SEM of the percentage of ^{45}Ca released into the medium during 5 days in culture for three to six pairs of bones. hPTHrP-(107-139) and sCT were both added at 10^{-8} M.

[a] Significantly different from contralateral control by paired *t* test, $P < 0.05$.

Cocultures of osteoclasts and UMR 106-01 cells

In coculture experiments, a biphasic response was noted with recombinant hPTHrP-(1–141). Concentrations of 10^{-11} and 10^{-10} M stimulated resorption, although this effect was progressively lost at the higher concentrations of 10^{-9} and 10^{-8} M (Fig. 2A). Furthermore, in the cocultures, both hPTHrP-(1–141) (10^{-8} M; Fig. 2B) and PTHrP-(107–139) (10^{-11}–10^{-9} M) (Fig. 2C) completely inhibited resorption stimulated by hPTH(1–34) (10^{-9} M).

Long term isolated osteoclast cultures

Osteoclasts became refractory to sCT inhibition when the culture period was extended to 96 h (Fig. 3), whereas inhibition by hPTHrP-(107–139) was maintained. This escape phenomenon seen with CT has long been recognized from observations made in bone organ culture (26).

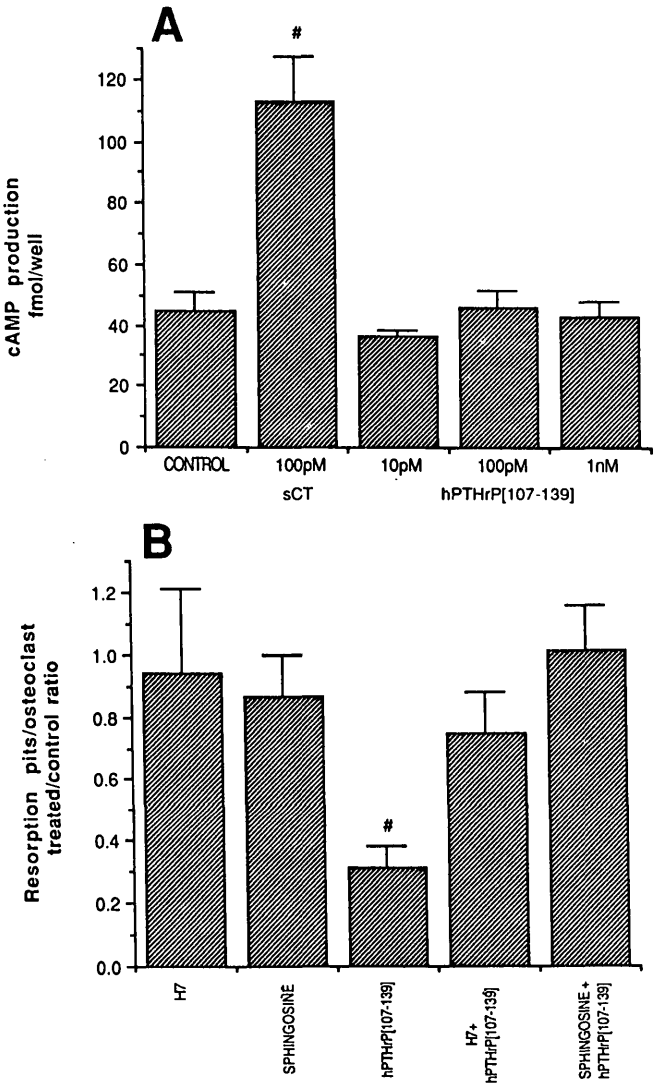

FIG. 5. A, cAMP response of osteoclast cultures to treatment with hPTHrP-(107–139) and sCT. No cAMP response to hPTHrP-(107–139) (10^{-15}-10^{-9} M) was seen in three other similar experiments. The osteoclast count per well was 115 ± 5 (mean ± SEM). #, $P < 0.001$. B, Effects of H7 and sphingosine on the response of osteoclasts to hPTHrP-(107–139) (10^{-9} M). Osteoclasts cultured on bone slices were treated with H7 (5×10^{-6} M), a protein kinase-A and -C inhibitor (28), or sphingosine (10^{-6} M), an inhibitor of protein kinase-C (27), alone or concurrently with hPTHrP-(107–139) (10^{-9} M). #, $P < 0.001$, compared to osteoclast cultures treated with inhibitors alone or inhibitor plus hPTHrP-(107–139).

Osteoclast spreading assay

The osteoclast cytoplasmic spreading assay was refined from methods previously described (20–22). By dividing the plan area of each osteoclast by its number of nuclei, the precision of the assay was significantly increased. When purified osteoclasts were, thus, cultured on glass chamber slides, hPTHrP-(107–139) reduced osteoclast cytoplasmic spreading at concentrations of 3×10^{-15} M and above (Fig. 4A). This effect was evident 30 min after treatment with hPTHrP-(107–139) (10^{-11} M) and persisted for at least 7 h (Fig. 4B).

Organ culture assay

Organ culture studies using a fetal rat long bone assay (24) demonstrated that hPTHrP-(107–139) inhibited basal resorption to a similar extent as sCT (Table 1).

Signal transduction pathways

The signal transduction mechanisms operating for PTHrP-(107–139) appear to be distinct from those for CT. There was no elevation of cAMP in response to hPTHrP-(107–139) in the presence of a 2.5-fold response to sCT (Fig. 5A). Sphingosine, a protein kinase-C and calmodulin kinase inhibitor (27), and 1-(5-isoquinolinesulfonyl)2-methyl piperazine (H7), a protein kinase-A and protein kinase-C inhibitor (28), completely blocked the inhibitory effect of hPTHrP-(107–139) (Fig. 5B). One interpretation of these results is that protein kinase-C may play a significant role in mediating the effect of PTHrP-(107–139) on osteoclasts. Consistent with this, recent studies have shown that the protein kinase-C stimulator, 12-*O*-tetradecanoylphorbol-13-acetate, inhibits bone resorption *in vitro* in a manner which suggests a direct effect on osteoclasts (29). The inorganic calcium channel blocker nickel chloride (2.5×10^{-5} M) and the organic calcium channel blockers verapamil (10^{-5} M) and nifedipine (10^{-5} M) failed to inhibit the effect of PTHrP-(107–139) on bone resorption (results not shown).

Discussion

Our results indicate that the carboxyl-terminal portion of PTHrP is a potent inhibitor of bone resorption in the purified osteoclast, the osteoclast-osteoblast coculture, and the bone organ culture systems. Furthermore, PTHrP-(107–139) produces a rapid inhibition of cytoplasmic spreading in isolated osteoclasts. This response is similar to that seen with other agents, including CT and prostaglandin E_2, which are known to directly inhibit osteoclast function (20, 21). This result and those of the purified osteoclast bone resorption assay suggest that PTHrP-(107–139) acts directly on osteoclasts.

In the purified osteoclast bone resorption assay, the potency of hPTHrP-(107–139) appears to be 3–4 orders of magnitude greater than that of the full-length molecule. It seems likely that PTHrP-(1–141) requires proteolytic processing for full activity. A series of five basic residues, in the sequence Lys Lys Lys Arg Arg immediately preceding Thr^{107}, provides sites for potential processing. The lack of effect of Tyr^{106}-hPTHrP-(106–139) is consistent with the likelihood that the processing of PTHrP-(1–141) at Arg^{106} is required to generate a bio-

logically active carboxyl-terminal peptide.

The biphasic response seen with PTHrP-(1–141) in the osteoclast-osteoblast cocultures is consistent with dual opposing actions of the molecule on the two cell types present in the cocultures: stimulation of resorption via interaction of the amino-terminus with osteoblast PTHrP receptors (6) and inhibition via a direct effect of the carboxyl-terminal fragment with osteoclasts. The results of studies by others with full-length PTHrP-(1–141) are also consistent with the molecule possessing inhibitory as well as stimulatory effects. In intact bone organ culture, hPTHrP-(1–141) is 100-fold less potent than hPTH-(1–34) despite being 10-fold more potent in stimulating adenylate cyclase in osteoblast-like cells (30). Furthermore, hPTHrP-(1–141) appears to be significantly less potent than Tyr^{36}-hPTHrP-(1–36) in inducing hypercalcemia in intact rats (30, 31).

There now appear to be three distinct regions of the PTHrP molecule with different biological activities. The PTH-like activity of the amino-terminal 34 residues, PTHrP-(1–34), is well established, and we have previously shown that the effect of hPTHrP-(1–141) to promote placental calcium transport in the sheep requires the midmolecular sequence between residues 35–108 (9, 10). The present findings demonstrate potent osteoclast inhibitory effects between residues 107–139. Thus, PTHrP is reminiscent of the properties of the POMC molecule (32), which also acts as a precursor for peptides with differing biological activities.

PTHrP has been localized by immunohistology in bone cells in areas of endochondral and intramembranous bone formation in the human fetus (33). Resorbing fetal rat long bones release PTHrP into the medium (34), and evidence has also been presented for production of a bone resorption inhibitor by such cultures (35). Thus, PTHrP could be produced in bone and processed to amino-terminal and carboxyl-terminal fragments with opposing actions. The demonstration of circulating carboxyl-terminal PTHrP in some patients with cancer (8) raises the possibility of a systemic role, modulating the bone-resorptive effect of amino-terminal PTHrP. The potential dual regulation by PTHrP adds a new dimension to our understanding of the endocrine and paracrine control of bone resorption. Identification of these regions and preparation of corresponding synthetic peptides now make it possible to explore individual regulatory processes in detail. It will be of great interest to determine whether carboxyl-terminal PTHrP has a role in conditions of increased bone resorption, including osteoporosis, Paget's disease, and the skeletal complications of cancer.

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