

## Parathyroid hormone receptors in avian bone cells

(cyclic AMP/calcium homeostasis/cultured osteoblasts/chicken calvaria)

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**ABSTRACT** We have demonstrated binding of synthetic bovine parathyroid hormone (1-34) [bPTH-(1-34)] to embryonic avian bone cells in monolayer culture. The binding sites have qualitative and quantitative characteristics of a physiologically important parathyroid hormone (PTH) receptor. At apparent steady state (60 min at 24°C), 5-10% of electrolytically labeled, receptor-purified <sup>125</sup>I-labeled bPTH-(1-34) bound specifically to the cells whereas nonspecific binding was less than 1% of the added labeled hormone. Scatchard analysis showed a single order of PTH binding sites ( $K_d = 0.6$  nM) with approximately 10,000 sites per cell. In this bone cell system, PTH bound to its binding site and stimulated cAMP accumulation over the same concentration range. Bovine PTH-(1-84) bound to the cells with the same apparent affinity as bPTH-(1-34).

Parathyroid hormone (PTH) is believed to exert its effects on mineral homeostasis by influencing the movement of calcium and phosphate into and out of the exchangeable mineral compartment of bone and by regulating mineral handling and 1,25-dihydroxy vitamin D<sub>3</sub> formation by the kidney. The hormone acts on these tissues at least in part by stimulating adenylate cyclase in the target cell membrane to increase intracellular cAMP levels (1). PTH increases the cAMP content of cultured murine (1) and chicken (2) bone cells, and PTH receptors have been characterized in bovine (3, 4), avian (5), canine (6), and human (6) kidney membranes. To date, however, the direct demonstration and quantification of skeletal PTH receptors by radioligand binding assay has not been accomplished.

The present study demonstrates the presence of specific, high-affinity PTH binding sites on cultured embryonic chicken bone cells. Characterization of these binding sites by using electrolytically labeled, receptor-purified bovine PTH-(1-34) reveals that they display properties consistent with the possibility that they function as hormone receptors coupled to adenylate cyclase.†

### METHODS

We obtained fertilized chicken eggs from Feather Hill Farms (Petaluma, CA) and incubated them in our laboratory. We obtained cell culture supplies from GIBCO, 35-mm tissue culture dishes from Becton Dickinson (Oxnard, CA), collagenase (lot 40C095, 148 units/mg) from Millipore Corporation (Worthington Division, Freehold, NJ), bovine serum albumin (fatty acid-free) from Miles, and Na<sup>125</sup>I from New England Nuclear. Synthetic human PTH-(1-34) [hPTH-(1-34); synthesized according to the sequence of Brewer *et al.* (7)] and human calcitonin were donated by Werner Rittel (Ciba Geigy Company, Basel, Switzerland) and bovine PTH-(1-34) (bPTH; 6000 units/mg) was

donated by Beckman. We obtained porcine insulin from Schwarz/Mann (Orangeburg, NY) and glucagon from Sigma.

**Bone Cell Isolation and Culture.** We used the embryonic chicken bone cell culture method developed by Peck *et al.* (8) as adapted for chicken bone cell culture by Nijweide *et al.* (2), but we did not use chicken embryo extract in the medium. Forty calvaria yielded 12-20 × 10<sup>6</sup> cells at initial count with a hemocytometer. Cells reached confluence and were used for experiments after 6-7 days of growth.

**Binding Studies.** Electrolytic labeling and receptor purification of bPTH-(1-34) were accomplished as described (3). The specific activities of the preparations of <sup>125</sup>I-labeled bPTH-(1-34) [<sup>125</sup>I-bPTH-(1-34)] used ranged from 75 to 100 μCi/μg (1 Ci = 3.7 × 10<sup>10</sup> becquerels). Culture medium was replaced with 0.5 ml of medium 199 containing 20 mM Hepes and 0.1% bovine serum albumin at pH 7.4. <sup>125</sup>I-bPTH-(1-34) and an appropriate amount of unlabeled hormone or diluent (10 mM acetic acid/0.1% albumin) were added, each in a volume of 10 μl. Incubations were carried out at room temperature (24°C). The monolayers were washed four times with 0.9 ml of Hanks' solution at 4°C. Then 0.9 ml of Hanks' solution was added, and the cells were scraped from the dishes with a rubber policeman and transferred to test tubes with a Pasteur pipette. Cell-bound and medium radioactivity were measured in a well-type gamma counter. Dishes containing no cells were incubated and washed in the same manner and the binding values obtained (≈0.5%) were subtracted from the total binding per culture. Protein was measured by the Lowry method (9), and binding was expressed as the ratio of bound to free hormone per mg of cell protein.

**cAMP Measurement.** cAMP accumulation was studied under conditions identical to those used in the binding experiments except that the medium also contained 1 mM 3-isobutyl-1-methylxanthine. Control experiments demonstrated that 1 mM 3-isobutyl-1-methylxanthine did not affect <sup>125</sup>I-bPTH-(1-34) binding. Cultures were incubated for 5 min. cAMP was extracted from the monolayers with three 1-ml washes of absolute ethanol, and the alcohol was evaporated in a Brinkman sample concentrator. cAMP was measured by the competitive protein binding assay of Gilman (10) and expressed as pmol of cAMP per culture (the ethanol treatment prevented accurate protein measurement). Addition of phosphodiesterase decreased the measured cAMP to undetectable levels, and dilution of extracted cAMP resulted in a dose-displacement curve parallel to that obtained with cAMP standard.

Abbreviations: PTH, parathyroid hormone; hPTH and bPTH, human and bovine PTH.

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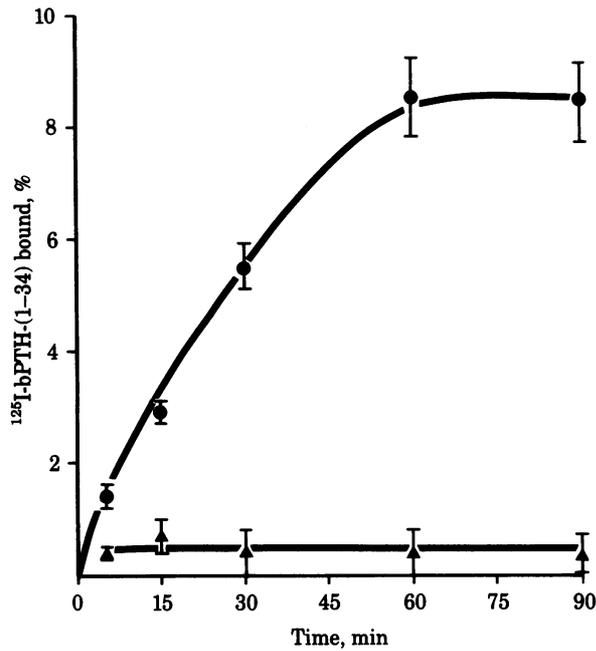


FIG. 1. Time course of specific (●) and nonspecific (▲)  $^{125}\text{I}$ -bPTH-(1-34) binding to cultured chicken bone cells. Each point represents the mean  $\pm$  SEM of values obtained in three separate experiments, corrected to 1 mg of cell protein. Nonspecific binding values were obtained in the presence of 243 nM unlabeled bPTH-(1-34).

## RESULTS

The bone cells tended to grow in clusters, with an average of  $2.5 \times 10^6$  cells, or 250  $\mu\text{g}$  of cell protein, per dish at confluence. Ninety percent or more of the cultured cells excluded trypan blue dye, indicating their gross viability. Electron micrographs revealed that both freshly isolated and cultured cells contained single nuclei and well-developed rough endoplasmic reticulum.  $^{125}\text{I}$ -bPTH-(1-34) bound specifically to chicken bone cells in

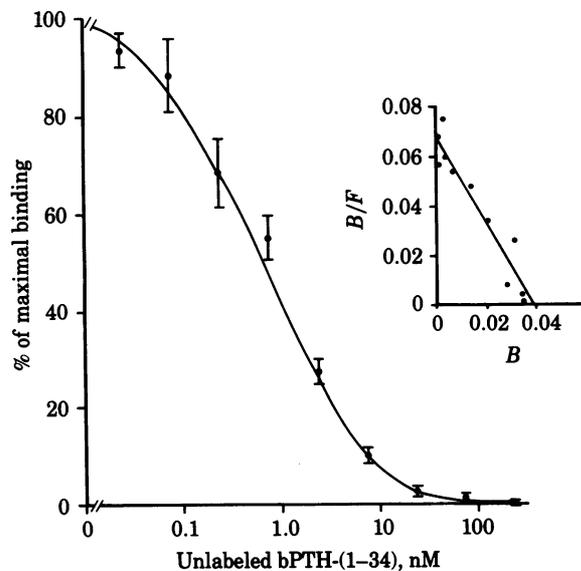


FIG. 2. Competitive inhibition of  $^{125}\text{I}$ -bPTH-(1-34) binding by unlabeled bPTH-(1-34) at apparent steady state. Each point represents the mean  $\pm$  SEM of normalized values obtained in five separate experiments. Total binding per culture ranged from 4% to 10% before normalizing. (Inset) Scatchard plot from a representative experiment. B, pmol per culture.

monolayer culture (Fig. 1). At 24°C, binding reached apparent steady state after 60 min and remained constant until 90 min. Longer incubations resulted in decreased binding. Nonspecific binding—that which occurred in the presence of 243 nM ( $1 \mu\text{g}/\text{ml}$ ) unlabeled bPTH-(1-34)—was  $\approx 0.5\%$  of the total added  $^{125}\text{I}$ -bPTH-(1-34). Specific binding per culture, before correction to 1 mg of cell protein, ranged from 5% to 10% of total. To assess reversibility of binding, 243 nM unlabeled bPTH-(1-34) was added to cultures that had already been incubated for 60 min with labeled bPTH-(1-34) alone. At 90 min, specific binding was decreased by 60%; 40% of the labeled material appeared to be irreversibly bound to the monolayers.

Competitive binding studies carried out at apparent steady

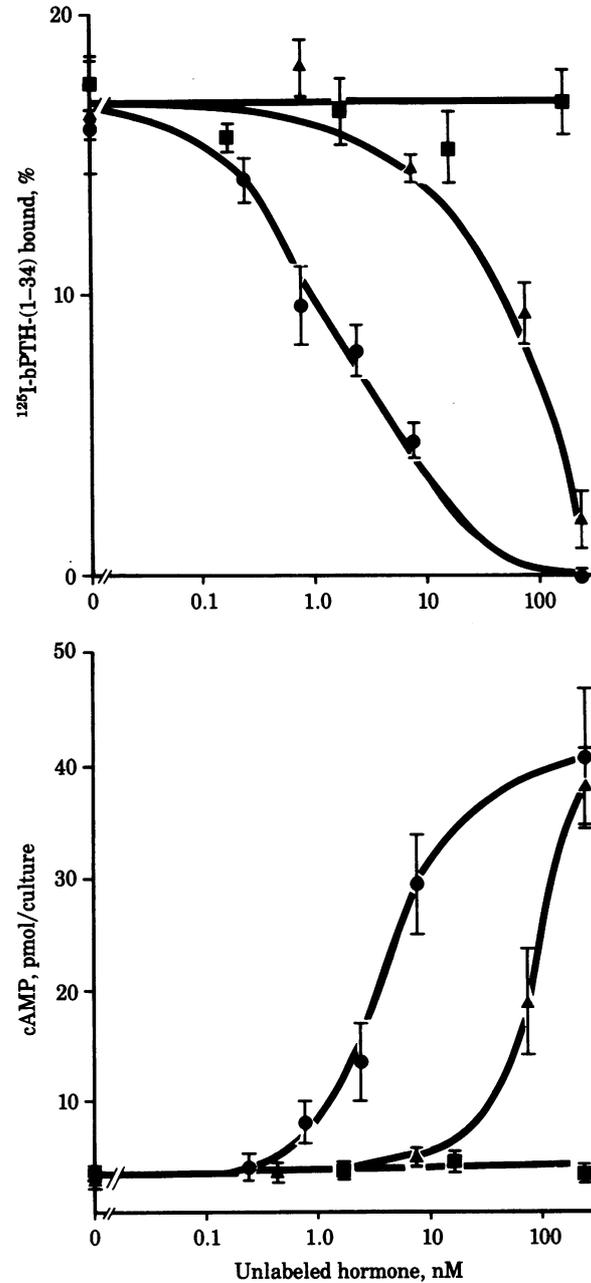


FIG. 3. (Upper) Competitive inhibition of  $^{125}\text{I}$ -bPTH-(1-34) binding at apparent steady state. (Lower) Stimulation of cAMP accumulation in chicken bone cells. ●, Unlabeled bPTH-(1-34); ▲, hPTH-(1-34); ■, porcine insulin. Each point represents the mean  $\pm$  SD of triplicate determinations from a single, representative experiment. Binding values are expressed per mg of cell protein.

state revealed that half-maximal reduction in  $^{125}\text{I}$ -bPTH-(1-34) binding occurred in the presence of  $\approx 1$  nM unlabeled bPTH-(1-34) (Fig. 2). Complete inhibition of specific  $^{125}\text{I}$ -bPTH-(1-34) binding was obtained in the presence of 25–75 nM unlabeled hormone. Decreased  $^{125}\text{I}$ -bPTH-(1-34) binding was often observed in the presence of as little as 75 pM unlabeled hormone. Scatchard analysis of apparent steady-state data from six experiments revealed a mean ( $\pm$  SEM)  $K_d$  of  $0.6 \pm 0.1$  nM and approximately 10,000 sites per cell, assuming equal distribution of binding sites among the cell population.

PTH-(1-34) stimulated cAMP accumulation over the same concentration range as it inhibited  $^{125}\text{I}$ -bPTH binding (Fig. 3). Half-maximal cAMP stimulation occurred in the presence of about 3 nM bPTH-(1-34); 243 nM bPTH-(1-34) evoked a 10-fold increase in cAMP.

PTH-(1-34) synthesized according to the sequence of Brewer *et al.* (7), found to be less active than bPTH-(1-34) in renal receptor systems (3), was 1/30th as potent in binding and in stimulation of cAMP accumulation (Fig. 3). bPTH-(1-84) displaced  $^{125}\text{I}$ -bPTH-(1-34) as effectively as unlabeled bPTH-(1-34). In these experiments, half-maximal displacement of  $^{125}\text{I}$ -bPTH-(1-34) occurred in the presence of 1.0 nM bPTH-(1-34) and 1.1 nM bPTH-(1-84). Insulin did not displace labeled PTH or stimulate cAMP accumulation at concentrations up to 170 nM (1  $\mu\text{g}/\text{ml}$ ). At 1.7  $\mu\text{M}$ , insulin displaced 10% of the labeled hormone. Glucagon and human calcitonin did not compete with  $^{125}\text{I}$ -bPTH for binding. Human calcitonin at 220 nM also did not influence cAMP levels.

## DISCUSSION

We have described an embryonic bone cell culture system in which we have measured specific binding of electrolytically labeled, receptor-purified  $^{125}\text{I}$ -bPTH-(1-34). The technique used should provide the means to evaluate directly the initial events in PTH action on the skeleton.

Although it is likely that the bone cell cultures we used contain more than one population of bone cells, electron micrographs of both freshly isolated and cultured bone cells showed cells with single nuclei and extensively developed rough endoplasmic reticulum. This observation and the fact that PTH stimulated cAMP accumulation in the cultures, but calcitonin did not, suggest that most of the cells were "osteoblast-like" (11).

Scatchard analysis was performed to estimate the affinity and number of PTH binding sites on the bone cells. The observed steady-state binding may not represent chemical equilibrium, as assumed in Scatchard analysis, because the labeled hormone may have been internalized, degraded, or irreversibly bound by the cells. Therefore, the calculated  $K_d$  and number of binding sites must be considered approximations. Based on the  $K_d$  derived, however, chicken bone cells bind bPTH-(1-34) with an affinity 5- to 10-fold greater than do isolated chicken renal plasma membranes (5). Furthermore, our estimate of the number of binding sites per cell is based on the assumption that the binding sites are uniformly distributed among the cell population, but this has not been demonstrated.

Previous studies of PTH receptor binding in bovine (4), chicken (5), canine (6), and human (6) renal plasma membranes and in isolated chicken renal tubules (12) showed that the dose range over which unlabeled PTH inhibited the binding of labeled PTH was similar to that which either stimulated adenylate cyclase (membranes) or stimulated the accumulation of cAMP (tubules). Although it is possible that coincidence plays a role

in the consistency of this relationship, we now find that it holds also for a different target of PTH, the bone cell. Because all of the tissues we have examined for PTH receptors and PTH stimulation of adenylate cyclase or cAMP accumulation have been derived from normal animals or humans, we have tentatively concluded that (i) a direct relationship between PTH receptor occupancy and adenylate cyclase activation probably exists under physiologic conditions and (ii) the presence of this relationship *in vitro* may be an important reflection of a normal state of coupling of the PTH receptor and adenylate cyclase. The fact that a less-active analog of PTH (6), hPTH-(1-34) synthesized according to the sequence of Brewer *et al.* (7), showed similar potency in both the binding and cAMP systems further supports the presence of this relationship of receptor occupancy and cAMP generation in the bone cells.

A body of evidence supports the idea that native PTH-(1-84) must be cleaved to shorter fragments to act on bone tissue (13). The similarity of the abilities of bPTH-(1-34) and bPTH-(1-84) to displace labeled bPTH-(1-34) is consistent with the interpretation that the two polypeptides bind to chicken bone cells with equal affinity and therefore does not support the idea that the native hormone must be cleaved to act on avian bone. However, cleavage of bPTH-(1-84) to release active amino-terminal fragments may have occurred during the 1-hr incubation period. We have not yet assessed the degree to which chicken bone cells in monolayer culture metabolize labeled or unlabeled bPTH-(1-34) or bPTH-(1-84).

PTH responsiveness may be altered in various physiologic and pathologic states. Whether such changes involve the PTH-receptor interaction itself or postreceptor modification has not been evaluated. The ability to quantitate skeletal PTH receptors will permit direct study of the initial events in PTH action on bone and may provide answers to such questions.

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