

Parathyroid Hormone Receptor in Intact Embryonic Chicken Bone: Characterization and Cellular Localization

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ABSTRACT The specific localization and the characterization of the parathyroid hormone (PTH) receptor in bone have been studied using 18-d embryonic chick calvariae and biologically active, electrolytically labeled [125 I] bovine PTH(1-34). Binding was initiated by adding [125 I]-bPTH(1-34) to bisected calvariae at 30°C. Steady state binding was achieved at 90 min at which time 10 mg dry wt of calvaria specifically bound 17% of the added [125 I]bPTH(1-34). Nonspecific binding in the presence of 244 nM unlabeled bPTH(1-34) was <2%. Insulin, glucagon, and calcitonin (1 μ g/ml) did not compete for PTH binding sites. Half-maximal inhibition of binding was achieved at concentrations of unlabeled bPTH(1-34) or bPTH(1-84) of about 10 nM. The range of concentration (2-100 nM) over which bPTH(1-34) and bPTH(1-84) stimulated cyclic 3',5'-adenosine monophosphate (cAMP) production was similar to that which inhibited the binding of [125 I]bPTH(1-34). Light microscope autoradiograms showed that grains were concentrated over cells (osteoblasts and progenitor cells) at the external surface of the calvariae and in trabeculae. In the presence of excess unlabeled PTH, labeling of control autoradiograms was reduced to near background levels. No labeling of osteocytes or osteoclasts was observed. At the electron microscopic level, grains were localized primarily over cell membranes. A quantitative analysis of grain distribution suggested that cellular internalization of PTH occurred.

Parathyroid hormone (PTH) acts directly on bone acutely to increase bone resorption and decrease bone formation (1, 2) and chronically to increase both bone resorption and bone formation (3). These actions are mediated by the cellular elements of bone by mechanisms involving, at least in part, the stimulation of cyclic 3',5' adenosine monophosphate (cAMP) production (4, 5). The evidence available indicates that PTH directly or indirectly influences all bone cells (osteogenic precursors, osteoblasts, osteocytes, and osteoclasts) (6-9); however, recent reports (10, 11) which describe the effect of PTH on cAMP production in osteoblastlike or osteoclastlike cells in monolayer culture implicate the osteoblast as the major osseous target cell of PTH.

During the past five years, major advances in the production of biologically active, high specific-activity, radioiodinated preparations of PTH have made it possible to investigate the previously elusive PTH receptor in kidney (12). It is only recently that similar progress has been made in the identification and characterization of PTH receptors in bone: using electrolytically [125 I]-labeled, receptor-purified, synthetic bo-

vine PTH tetratriacontapeptide [bPTH(1-34)] as ligand, we demonstrated high affinity, specific hormone binding sites in mixed monolayer cultures of chick embryonic bone cells which correlated directly with PTH-stimulated cAMP production in the same cultures (13).

The purpose of this investigation was to identify the bone cells in the frontal bones of chick embryonic calvariae that bind bPTH(1-34) in vitro and to determine the biochemical characteristics of this binding. Light microscope autoradiograms of calvariae after exposure to [125 I]bPTH(1-34) showed predominant localization of grains over lining cells (osteoblasts and osteogenic precursor cells) and the cell processes of osteoblasts undergoing transformation to osteocytes. Electron microscope autoradiograms showed predominant localization of grains over cell membranes. A quantitative analysis of grain distribution suggested that significant cellular internalization of PTH is a result of the receptor binding of this hormone to bone cells.

The results of biochemical studies of the binding of [125 I]-bPTH(1-34) to calvariae in vitro showed rapid incorporation

into calvariae which was competitively inhibited by unlabeled bPTH(1-34) and bPTH(1-84). The range of concentration over which binding was inhibited by unlabeled bPTH(1-34) was similar to that which stimulated cAMP production, suggesting a close relationship between these two events.

MATERIALS AND METHODS

Reagents

We obtained Na¹²⁵I from New England Nuclear (Boston, MA), porcine insulin from Schwarz/Mann (Div. Becton, Dickinson & Co., Orangeburg, NY), glucagon (extracted from porcine and bovine pancreas) and phosphodiesterases from Sigma Chemical Co. (St. Louis, MO), [⁸Nle, ¹⁸Nle, ³⁴Tyr] bovine PTH(3-34)amide (bPTH[3-34]amide) (14, 15) from Peninsula Laboratories, Inc. (San Carlos, CA). Synthetic human PTH(1-34) (synthesized according to the sequence of Brewer et al. [16]), (hPTH[1-34]B) and bovine PTH(1-34), (bPTH[1-34]), 6000 U/mg) were donated by Beckman Bioproducts (Palo Alto, CA), salmon calcitonin (4500 IU/mg) by Armour Pharmaceutical Company (Kankakee, IL), and bovine bPTH(1-84) (bPTH[1-84]), 2500 U/mg) by Dr. Bryan Brewer (National Institutes of Health, Bethesda, MD).

Embryonic Calvariae

Fertilized chicken eggs (Feather Hill Farms, Petaluma, CA) were incubated at 37°C until day 18. Frontal bones of calvariae were then removed and dissected free of surrounding connective tissue (including periosteum). Isolated half calvariae were washed (2 ml × 3) in Hanks' balanced salt solution (HBSS) containing 20 mM HEPES and equilibrated for 30 min. at 30°C in 2 ml of incubation medium (MEM containing 20 mM HEPES and 0.1% BSA, fatty acid free, Miles Laboratories, Inc., Elkhart, IN). All experiments were performed after the 30-min equilibration period.

Biochemical Binding Experiments

Biologically active, electrolytically iodinated, receptor-purified [¹²⁵I]bPTH(1-34) was prepared as previously described (12). For binding experiments, one-half calvaria was incubated in 0.5 ml of incubation medium at 30°C in a shaking incubator. Binding was initiated by adding 12–32 × 10⁻³ nM [¹²⁵I]bPTH(1-34) (50–130 pg, specific activity 85–100 μCi/μg) to the incubation medium containing an appropriate amount of unlabeled bPTH(1-34), other peptide hormones, or diluent (10 mM acetic acid, 0.1% BSA), each in a volume of 10 μl. Incubations were terminated by washing the calvariae with iced HBSS, 1 ml × 3 times). Each half calvaria was then counted in a well-type gamma scintillation counter (efficiency 80%) to determine the radioactivity bound. Binding was expressed as the percent of the total radioactivity added to incubation media that was bound to calvariae. Total binding was that which occurred in the presence of diluent; nonspecific binding was that which occurred in the presence of 244 nM unlabeled bPTH(1-34); specific binding was expressed as the total minus nonspecific binding. Three types of binding experiments were performed: (a) the time course of the total and non-specific binding of labeled bPTH(1-34), (b) the release of labeled bPTH(1-34) bound; labeled PTH was incubated with calvariae until steady state binding was achieved, at which time 244 nM unlabeled PTH(1-34) was added to the incubations, and binding was determined at various times thereafter, and (c) competitive binding assays; labeled bPTH(1-34) and various concentrations of either bPTH or other peptide hormones were added at the same time and binding was determined after 90 min of incubation. The peptide hormones tested were bPTH(1-34), bPTH(1-84), hPTH(1-34), the PTH antagonist, bPTH(3-34)amide, porcine insulin, salmon calcitonin, and porcine-bovine glucagon.

Assay of cAMP

Conditions used to measure cAMP accumulation were identical to those used in binding experiments except that the medium contained 1.0 mM 3 isobutyl-1-methyl xanthine (IBMX) (Sigma Chemical Co.). Control experiments demonstrated that 1.0 mM IBMX did not interfere with PTH binding. PTH peptides were added to the incubation medium in 10 μl of 10 mM acetic acid, 0.1% BSA, to give final concentrations ranging from 1.0–244 nM. After 15 min of incubation, half calvariae were washed and transferred into 1 ml of *N*-propanol and sonicated three times for 15 s after addition of (³H) cAMP (3000 cpm, 0.1 pmol) (New England Nuclear, Boston, MA) which was used as a recovery standard. Half calvariae were then removed from the sonicate and rinsed twice with 1 ml of *N*-propanol, and the washes were combined with the sonicate and centrifuged (15 min, 4,000 g). The supernatant was evaporated in a Brinkmann sample concentrator (Brinkmann Instruments, Inc., Westbury, NY). Samples were resuspended in 50 mM Na acetate, pH 4.0. Aliquots were assayed for (³H) cAMP (recovery,

70–90%), and cAMP was measured using the competitive protein binding assay of Gilman (17) and expressed as pmol cAMP/10 mg dry wt of calvaria. Added phosphodiesterase (1 mU/50 μl sample) reduced cAMP extracted from calvariae to undetectable levels. The dose-response curves produced by multiple dilutions of samples were linear and parallel to the standard curve produced by authentic cAMP.

The results (PTH binding, cAMP) are expressed per 10 mg dry wt of calvariae. Half calvaria weighed 7–9 mg and dry wt values correlated with DNA content (correlation coefficient $r = 0.83$). The mean DNA content of half calvaria was 25–40 μg.

Metabolism of PTH

For rebinding studies, media recovered from incubations of [¹²⁵I]bPTH(1-34) with calvariae at various times were incubated with freshly isolated calvariae for 60 min. Specific PTH binding to calvariae was then determined. Similarly derived medium was also chromatographed after 60-min incubations on Bio-Gel P-6 80 cm × 2 cm columns (Bio-Rad Laboratories, Richmond, CA) as described by Neuman et al. (18), except that 3 M acetic acid replaced formic acid in the 3-M guanidine elution buffer. Elution profiles of radioactivity were compared to those obtained when media containing [¹²⁵I]bPTH(1-34) which had been incubated without calvariae were chromatographed.

Autoradiographic Studies

Binding experiments for autoradiography were performed as described in the section entitled *Biochemical Binding Experiments*, except that ~10–15 times (120–555 × 10⁻³ nM) more [¹²⁵I]bPTH(1-34) was added per half calvaria. After three washes with iced HBSS, calvariae were cut with a razor blade into ~1 mm² pieces. These pieces were immersion-fixed for 2 h at 4°C in 2.5% glutaraldehyde, 0.7% paraformaldehyde in 0.2 M sodium bicarbonate buffer (S.B. buffer). After an overnight wash at 4°C in 0.2 M S.B. buffer the tissue was osmicated in osmium tetroxide containing 1.5% KCN for 2 h, rinsed in 0.2 M S.B. buffer, dehydrated in increasing concentrations of ethanol, and embedded in Epon-812. For light microscope autoradiography, 1-μm sections were placed on glass slides and coated with Kodak NTB-3 emulsion, and developed after 2–6 wk exposure (19). The light autoradiograms were used to determine the tissue blocks that contained sufficient radioactivity to perform electron microscope autoradiography. For electron microscope autoradiography, sections of 500 Å were placed on parlodion-coated grids, overlaid with a monolayer of Ilford-L-4 emulsion, exposed from 3–7 wk, developed, and stained with lead citrate (19, 20). Electron autoradiograms were photographed in a Philips 300 microscope at a magnification of 12,000.

Cell Internalization of Radioactivity

Grain distribution in cells observed in electron microscope autoradiograms was analyzed as described by Salpeter et al. (21) as adapted by Goldfine et al. (20). In this method, the distance from the center of grains to the nearest plasma membrane is measured with a graphic data digitalizer (Ladd Research Industries, Burlington VT). Statistical evaluation of the data was performed by chi-square analysis (22).

RESULTS

Characteristics of Binding of ¹²⁵I bPTH(1-34) to Calvariae

Binding of [¹²⁵I]bPTH(1-34) to added calvariae increased with time to reach an apparent steady state after 60 min. 10 mg dry wt of calvariae specifically bound 17% of the total [¹²⁵I]-bPTH(1-34) at 90 min (Fig. 1). Binding was maintained at this level for at least 1 h. Nonspecific binding of [¹²⁵I]bPTH(1-34) was <2% of the total added labeled hormone added at each time point. Addition of 244 nM unlabeled bPTH(1-34) after steady state binding of [¹²⁵I]bPTH(1-34) had been established (90 min) caused 50% of bound labeled hormone to be released from calvariae over a period of 60 min (Table I).

Bovine PTH(1-34) and bPTH(1-84) were equipotent in competitively inhibiting the binding of [¹²⁵I]bPTH(1-34) to calvariae (Fig. 2). Half-maximal inhibition of binding was achieved at concentrations of unlabeled hormone of about 10 nM. The competitive antagonist of PTH in vitro, [⁸Nle, ¹⁸Nle, ³⁴Tyr]bPTH(3-34)amide, [bPTH(3-34)amide], was

less effective than bPTH(1-34) and bPTH(1-84) in competitively inhibiting the binding of [¹²⁵I]bPTH(1-34) to calvariae; 100 nM bPTH(3-34)amide was required for half-maximal inhibition of binding. The preparation of human PTH(1-34), synthesized according to the amino acid sequence proposed by Brewer et al. (16), (hPTH[1-34]B), which has been found to be 30- to 140-fold less potent than bPTH(1-34) in several bioassay systems (23, 24), was less effective in inhibiting the binding of [¹²⁵I]bPTH(1-34), half-maximal inhibition occurring at ~1,000 nM. Salmon calcitonin, porcine-bovine glucagon, and porcine insulin were ineffective as inhibitors of [¹²⁵I]bPTH(1-34) binding to calvariae (Fig. 2).

Stimulation of cAMP Production in Calvariae by PTH Peptides

The time course for the stimulation of cAMP production in calvariae by 0.244 and 12 nM PTH is shown in Fig. 3. Peak responses were consistently observed at 15 min, and this time was selected to measure the responsivity of calvariae to various doses of PTH and other peptides. Bovine PTH(1-34) and bPTH(1-84) were equipotent in this regard (Fig. 4). The range of concentrations (2-100 nM) over which bPTH(1-34) stimulated cAMP in calvaria (Fig. 4) was similar to that which inhibited the binding of [¹²⁵I]PTH(1-34) (Fig. 2). As in the binding assay, hPTH(1-34)B was considerably less potent than bPTH(1-34) in the cAMP assay (Fig. 4). The PTH antagonist, bPTH(3-34)amide, did not stimulate calvarial cAMP production but did inhibit this effect by bPTH(1-34). The apparent inhibition constant of cAMP production (K_I) for bPTH(3-34)amide (73 nM), when a single near-maximal dose of bPTH(1-34) was assayed in the presence of various doses of bPTH(3-34)amide, was ~10 times higher than the apparent stimulation constant (K_S) for bPTH(1-34) (data not shown). This result is consistent with the difference in affinity noted in the inhibition of binding studies (Fig. 2).

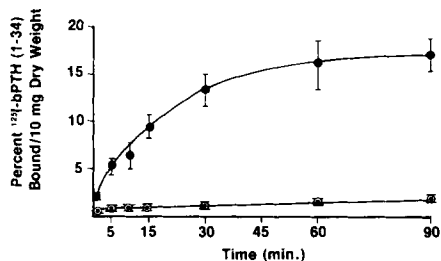


FIGURE 1 Time course of [¹²⁵I]bPTH(1-34) binding to calvariae at 30°C. Binding is expressed as percent of total radioactivity added per incubation bound to 10 mg dry wt of calvariae. Nonspecific and total binding are those that occurred in the presence and absence, respectively, of 244 nM unlabeled bPTH(1-34). Specific binding was calculated by subtracting nonspecific from total binding at each time point. Results are expressed as the mean value ±SD obtained in two experiments in which triplicate determinations were done. 17% represents ~2.30 fmol bound. (●) Specific, (○) Non-specific binding.

Metabolism of [¹²⁵I]bPTH(1-34) by Calvariae

We performed experiments of two types which strongly suggested that [¹²⁵I]bPTH(1-34) in the incubation medium was metabolized during incubation with calvariae. The first were

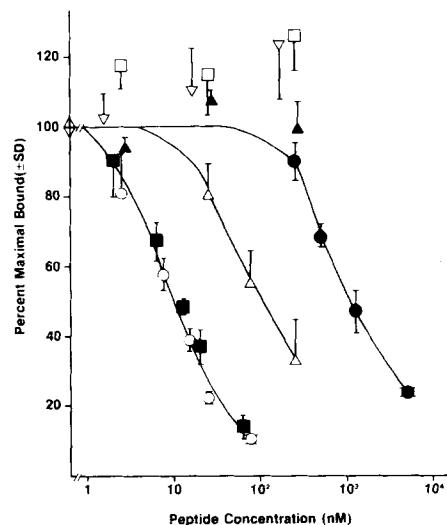


FIGURE 2 Competitive inhibition of [¹²⁵I]bPTH(1-34) binding by peptide hormones at 30°C. bPTH(1-34) (○), bPTH(1-84) (■), [⁸Nle,¹⁸Nle,³⁴Tyr]bPTH(3-34)amide (△), hPTH(1-34), Brewer sequence (●), calcitonin (□), insulin (▽), and glucagon (▲). Results are expressed as the mean values of three experiments performed in triplicate ±SD. Nonspecific binding was subtracted from all values. Binding is expressed as % maximal binding; 100% binding represents [¹²⁵I]bPTH(1-34) binding in the absence of unlabeled bPTH(1-34) (or 2.99 ± 0.47 fmoles).

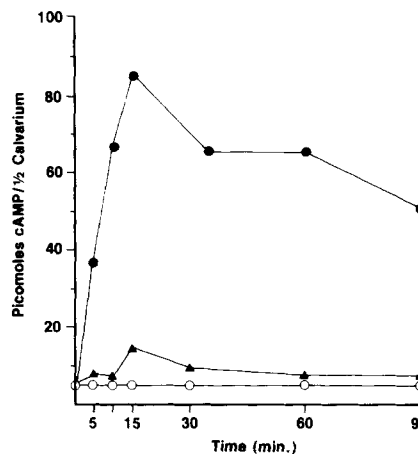


FIGURE 3 Time course for stimulation of cAMP production in calvariae by 1 ng (0.244 nM) (▲) and 50 ng (12 nM) (●) bPTH(1-34) at 30°C in the presence of 1 mM IBMX. cAMP was extracted as described in Materials and Methods and assayed by the method of Gilman (17). Basal levels (○) do not change with time. Data points are the mean values of duplicate determinations.

TABLE I
Release of Labeled bPTH (1-34) Bound to Calvariae

bPTH (1-34) 244 nM	0	15 min	30 min	60 min	90 min
B/T*/10 mg dry weight calvariae	22.25 ± 1.64	21.52 ± 2.47	16.53 ± 0.76	10.52 ± 0.22	10.21 ± 0.83

Labeled PTH was incubated with calvariae until steady state was achieved (90 min), at which time 244 nM unlabeled bPTH (1-34) was added to the incubations, and total binding was determined at times indicated. Results are the mean of one experiment in triplicate ±SD.

* B/T is the percent of the total radioactivity added to the incubation media that was bound to calvariae.

± 0.91 ± 0.01 fmol bound/10 mg dry weight calvariae.

studies of [125 I]bPTH(1-34) rebinding. After exposure of [125 I]bPTH(1-34) for 30 min at 30°C to calvariae, the specific binding of radioactivity to fresh calvariae was reduced by 50% compared to [125 I]bPTH(1-34) that had not been incubated with calvariae (Table II). Longer incubations of labeled hormone with calvariae further reduced specific binding of medium radioactivity to fresh calvariae.

The second experiments were investigations of the radioactive molecular components in media after incubation of [125 I]bPTH(1-34) with calvariae. After 60 min of incubation, the major portion of medium radioactivity eluted from gel filtration columns in a position consistent with a mol wt <1,000 (Fig. 5A). Such degradation of medium [125 I]bPTH(1-34) by calvariae was markedly inhibited by co-incubation of the labeled hormone with excess unlabeled bPTH(1-34) (244 nM) although labeled fragments of PTH were observed even under these conditions (Fig. 5B).

Autoradiographic Studies

Half calvariae were incubated for 60 min with 1.5×10^5 cpm [125 I]bPTH(1-34) alone (total binding) or with 244 nM unla-

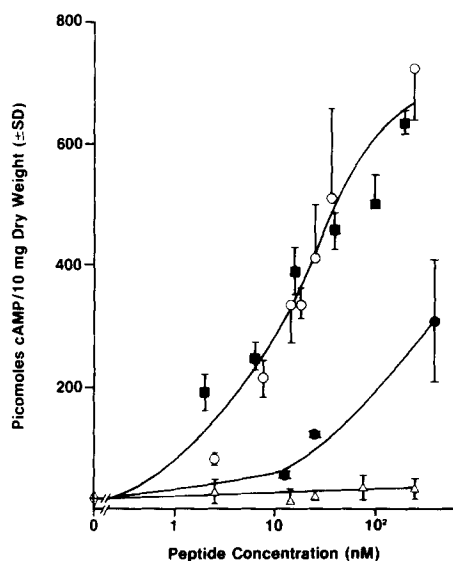


FIGURE 4 Stimulation of cAMP production in calvariae by PTH peptides at 30°C in the presence of 1 mM IBMX. cAMP was extracted as described in Materials and Methods. Results are expressed as the mean values \pm SD obtained in two experiments in which triplicate determinations were done. (○) bPTH(1-34), (■) bPTH(1-84), (△) bPTH(3-34), Amide Analogue, (●) hPTH(1-34), Brewer sequence.

TABLE II
Rebinding Experiments

Duration of the first incubation	% Specific binding after 60 min of incubation with freshly isolated calvariae
min	
0	100
30	50 ± 14.0
60	32 ± 6.5
90	20 ± 4.0

Media recovered from incubations of [125 I]bPTH(1-34) with calvariae at various times were incubated with freshly isolated calvariae for 60 min at 30°C. Specific PTH binding to calvariae was then determined. 100% specific binding (1.84 ± 0.10 fmol) is the specific binding of [125 I]bPTH(1-34) to calvariae after 60 min at 30°C without prior incubation of the labeled hormone with calvariae (mean \pm SE of two experiments in triplicate).

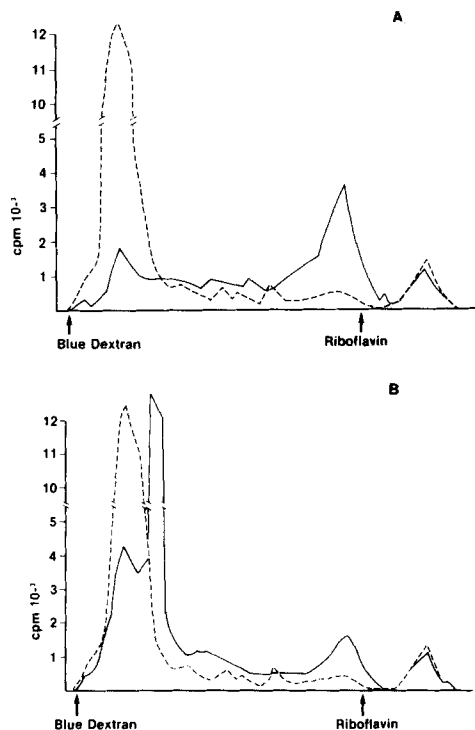


FIGURE 5 Gel filtration using Bio-Gel P6 and acetic acid-guanidine buffer (see Materials and Methods) of medium containing labeled bPTH(1-34) incubated for 60 min at 30°C in the absence of calvariae (-----, A and B) and in the presence of calvariae without and with 244 nM bPTH(1-34) (—, A and B, respectively). Blue dextran was used to determine the void volume, and riboflavin was used to mark mol wt 375.

beled PTH (nonspecific binding). At the end of incubation, total binding was 3.5×10^4 cpm, (30% of the added labeled hormone) and nonspecific binding was 2×10^3 cpm (1% of the added labeled hormone). Light microscope autoradiograms representing total (Fig. 6a and b) and nonspecific binding (Fig. 6c) are shown in the composite of Fig. 6. Autoradiograms were also processed after 10 min of incubation, at which time 1.1×10^4 cpm were bound.

Few, if any, grains were observed on the micrographs representing nonspecific binding (Fig. 6c). In micrographs representing total binding, most of the grains were found associated with osteoblasts both in the peripheral portion of the calvariae (Fig. 6a) and in trabeculae (Fig. 6b) and to osteoprogenitor cells on the peripheral portion of the calvariae (Fig. 6a). Osteoprogenitor cells were characterized by their elongated shape, their localization on the outside portion of osteoblasts on the peripheral portion of the calvariae, and the absence of a well-developed rough endoplasmic reticulum on the electron microscope pictures. Grains were also seen overlying uncalcified collagen. At the electron microscopic level, the grains seen overlying this osteid tissue were predominantly associated with cellular cytoplasmic processes originating from osteoblasts surrounded by uncalcified matrix (Fig. 6e). We did not observe labeling of osteoclasts after either 10 min or 60 min of incubation at the PTH concentrations used (Fig. 6d). It is to be noted that the mononuclear cells that are in this micrograph in the immediate vicinity of the osteoclasts are not labeled. However, in other micrographs, labeling of osteoblasts positioned on the surface of the bone behind apparently active osteoclasts was present.

Electron microscope autoradiography demonstrated that

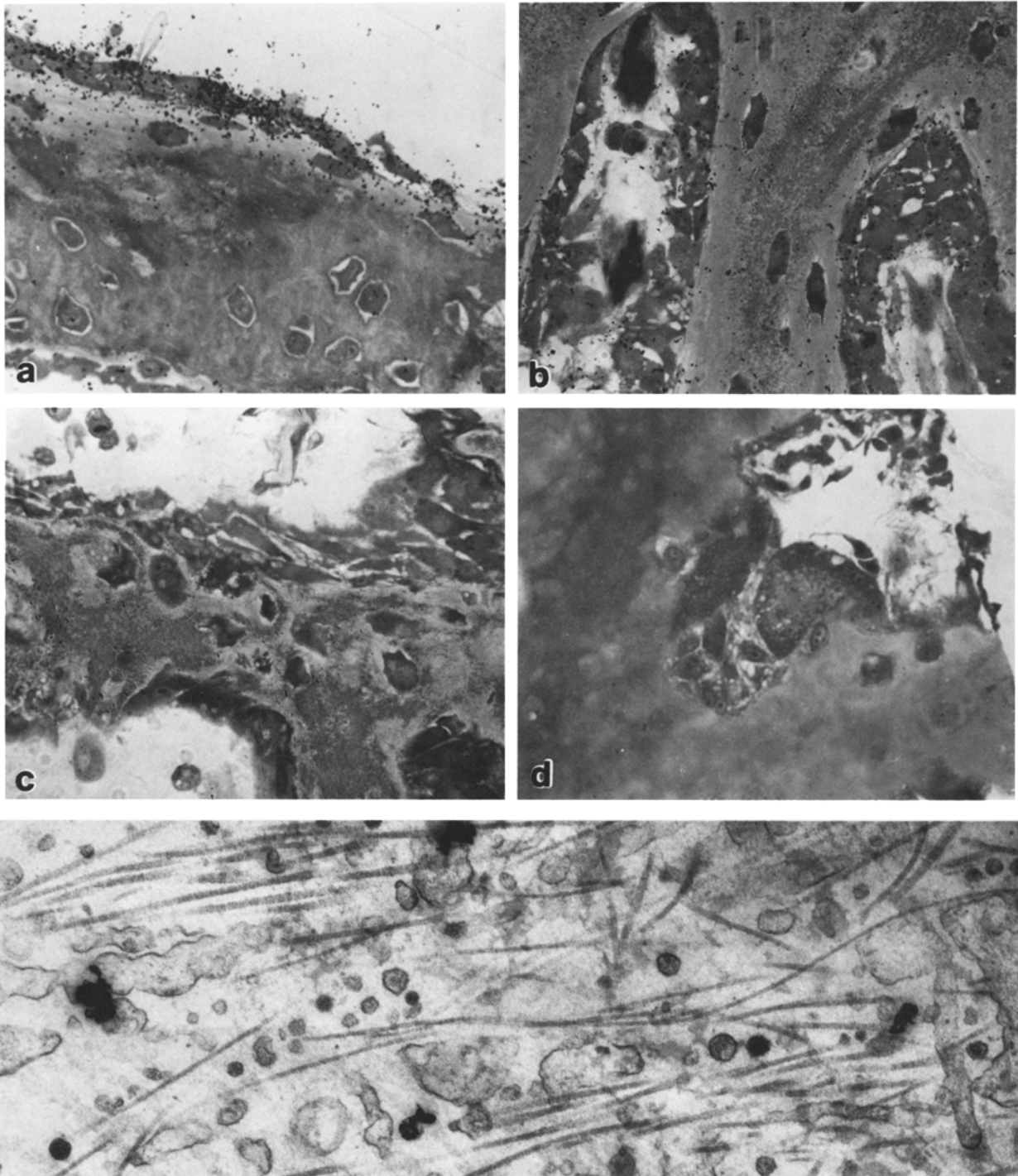


FIGURE 6 Micrographs of binding of [125 I]bPTH(1-34) to calvariae after 60 min of incubation at 30°C. (a, b) Light microscope autoradiograms of total [125 I]bPTH(1-34) binding to cells (osteoblasts and osteoprogenitor cells) at the peripheral portion of the calvariae (a) and to osteoblasts located inside of trabeculae (b) ($\times 450$). (c) Light microscope autoradiogram of nonspecific [125 I]bPTH(1-34) binding to trabeculae after 60 min. No grains are observed when 244 nM bPTH(1-34) is added together with the labeled PTH ($\times 450$). (d) Light microscope autoradiogram of [125 I]bPTH(1-34) binding to calvariae after 60 min, showing the absence of grain association with osteoclasts ($\times 450$). (e) Electron microscope autoradiogram of [125 I]bPTH(1-34) binding to calvariae after 60 min, showing that the grains seen overlying collagen at the light microscopic level (b) are associated with plasma membranes ($\times 34,000$).

after 60 min of incubation the grains were predominantly associated with the plasma membrane of osteoblasts (Fig. 7 a). Grains were also observed over coated pits (Fig. 7 b) and coated vesicles (Fig. 7 c). To determine whether grain distribution was consistent with the cellular internalization of radioactivity, we

performed a quantitative analysis of the grains overlying the cell. The expected distribution of grains overlying the cells, if all the radioactivity arose solely from 125 I bound to the plasma membrane, is shown in Fig. 8 A. One would expect 60% of the grains to be within 0.1 μ m of the plasma membrane, while only

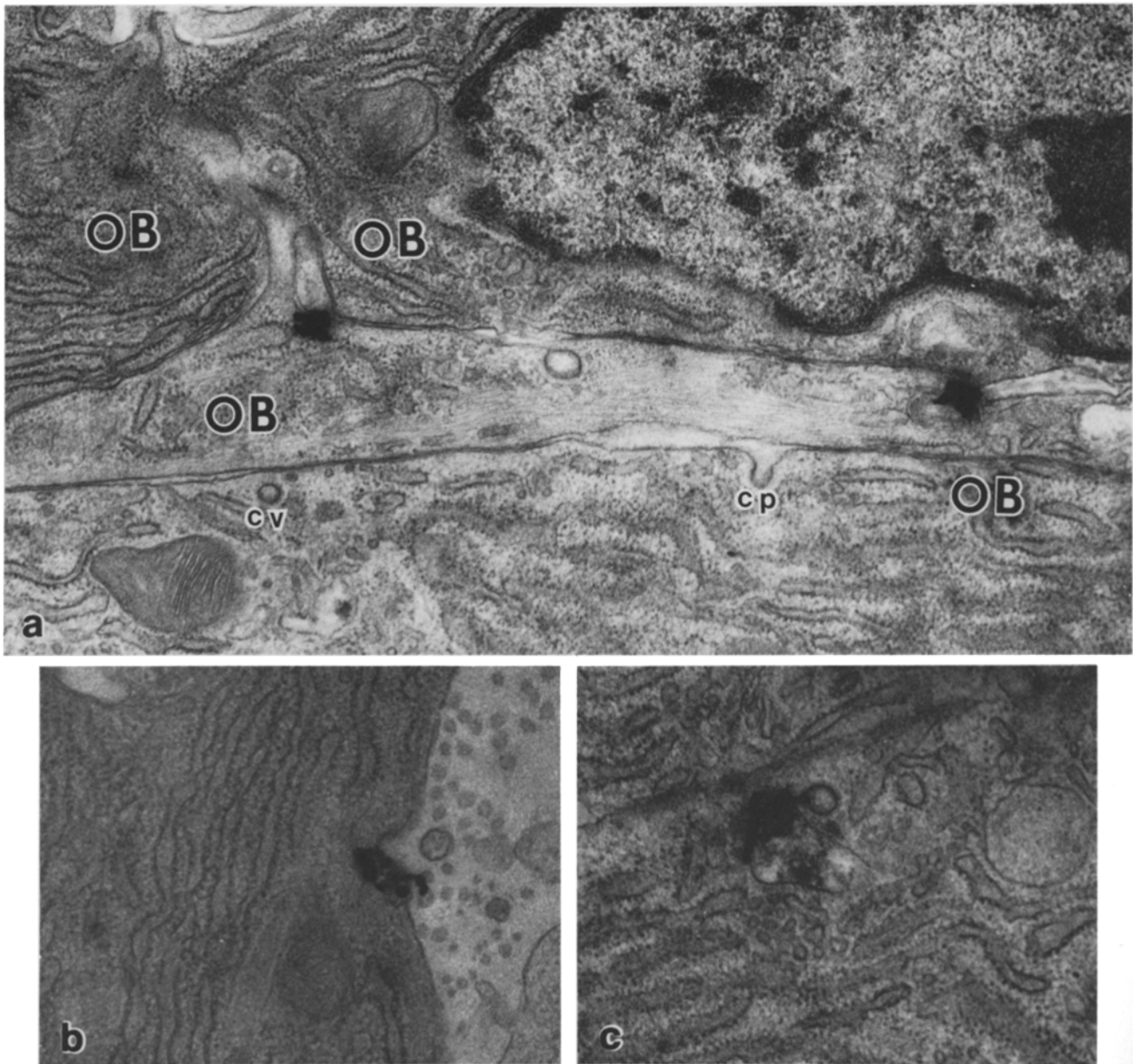


FIGURE 7 Electron microscope autoradiogram of [^{125}I]bPTH(1-34) total binding to calvariae after 60 min of incubation at 30°C ($\times 34,000$). (a) Association of grains with plasma membrane of osteoblasts (OB). (b, c) Association of grains with a coated pit (b) and a coated vesicle (c). Background was $<5\%$. (cp), coated pit; (cv) coated vesicle.

2% would be beyond $1\ \mu\text{m}$. The actual distribution of grains at a given distance from the plasma membrane found in our study is shown in Fig. 8B. Only 30% of the grains were within $0.1\ \mu\text{m}$ from the plasma membrane. Chi-square analysis of these data is consistent with significant internalization of radioactivity by osteoblasts ($P < 0.001$).

DISCUSSION

The present investigation was performed using intact calvariae, rather than isolated cultured bone cells, so as to observe [^{125}I]bPTH(1-34) binding to a full complement of the cellular elements of bone in their natural environment. Light microscope autoradiograms showed a surprisingly well-defined localization of radioactivity over osteoprogenitor cells, osteoblasts and the cell processes of osteoblasts in the process of being transformed into osteocytes. Nonspecific binding was

minimal. In contrast, we did not observe localization of radioactivity over osteocytes or osteoclasts. Although it is likely that these latter cells do not bind PTH, several alternative explanations are possible: (a) we used embryonic bone, and PTH receptors may only appear on osteoclasts and osteocytes after the embryo hatches; (b) our chances of observing localization of radioactivity over osteoclasts were diminished because of the paucity of these cells in chick calvariae; (c) the labeling of osteoclasts and osteocytes may have been precluded by poor penetration of incubation medium into calvariae; and (d) the affinity of PTH receptors on osteoclasts and osteocytes may be lower than that of those on osteoblasts so that, at the concentrations of [^{125}I]bPTH(1-34) used, labeling of these cells could not be detected.

After 60 min of incubation, most of the grains observed on electron microscopic autoradiograms were associated with the plasma membranes of osteoblastlike cells, consistent with the

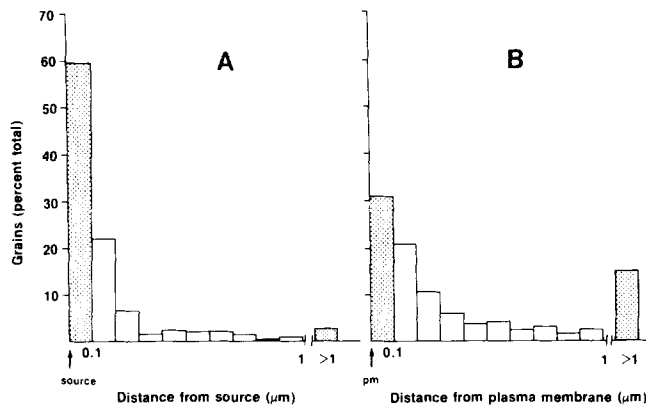


FIGURE 8 Grain distribution profiles for a line source of [^{125}I] (A) (289 grains counted) and for [^{125}I]bPTH(1-34) grains from the plasma membrane (B) (658 grains counted, 300 micrographs). Analysis of the grain distribution was performed following the method described by Salpeter et al. (21) (see Materials and Methods). (A) Distance between grains and a line source. (B) Distance between grains and plasma membrane.

presumed action of PTH via a membrane-bound receptor. However, quantitative analysis of the grain distribution overlying cells indicated that internalization of radioactivity occurred. Our data do not permit the conclusion that this "internalized" radioactivity was derived originally from that intact PTH bound to plasma membranes, but several ancillary observations suggest that this may be the case. Specifically, we observed grains associated with coated pits and vesicles, structures known in other systems (25, 26) to be involved in ligand-receptor complex internalization. Moreover, it is known, from studies on liver, that ^{125}I cannot be fixed during autoradiographic processing (27). Thus, it is likely that internalized radioactivity in our studies represents [^{125}I]bPTH(1-34) or a labeled fragment of this peptide that is being processed by the cell subsequent to membrane-associated receptor binding of the intact peptide. In contrast to the studies performed in renal tissue by Nordquist et al. (28) using chloramine T iodinated PTH which was probably biologically inactive, we did not identify a predominant localization of biologically active [^{125}I]PTH in mitochondria.

On the basis of our autoradiographic studies, it is reasonable to assume that osteoblastlike cells accounted for the major portion of the specific binding of [^{125}I]bPTH(1-34) we observed in calvariae incubated with this ligand. This binding was of high affinity; half-maximal inhibition of binding was achieved at a concentration of 10 nM unlabeled bPTH(1-34). This apparent affinity is similar to that found in chicken (12), dog (29), and human (29) renal plasma membranes but lower than that in isolated bone cells in culture (13). However, firm conclusions about the number of receptor types present on cells binding PTH in chick calvariae and the dissociation constant(s) of PTH from its receptor(s) cannot be drawn from these studies because the labeled PTH ligand we used was both internalized by cells and metabolized in the medium, and the ligand or a component thereof was irreversibly bound, as suggested by the acute displacement of only 50% of [^{125}I]bPTH(1-34) bound after 90 min of incubation with calvariae by added excess unlabeled bPTH(1-34).

These considerations aside, several of our observations suggest that the [^{125}I]bPTH(1-34) bound to calvariae represents biologically active hormone associated with a specific receptor: (a) the binding of [^{125}I]bPTH(1-34) was highly specific (only

unlabeled PTH but not other peptide hormones competitively inhibited binding); (b) there was close correspondence between the ability of various PTH peptides to competitively inhibit binding and stimulate cAMP production; and (c) the products of the metabolism of [^{125}I]bPTH(1-34) by calvariae bound less well to fresh calvariae than [^{125}I]bPTH(1-34).

We found that bPTH(1-34) and bPTH(1-84) were equipotent in competitively inhibiting the binding of [^{125}I]bPTH(1-34) and in stimulating cAMP production. Although this observation does not support the recent suggestion of Martin et al. (30), based on whole canine bone perfusion studies, that an amino-region fragment of PTH and not PTH(1-84) has osseous activity, it is possible that the binding and adenylate cyclase activity of bPTH(1-84) in our system was due to a biologically active fragment of the hormone produced by the metabolism of PTH(1-84) by calvariae.

In agreement with our previous investigations of isolated renal tubules (31), renal plasma membranes from the avian (12), canine (29) human (29), and avian cultured bone cells (13), we observed that the range of concentrations over which unlabeled bPTH(1-34) competitively inhibited binding of [^{125}I]bPTH(1-34) in embryonic avian calvariae corresponded closely with that which stimulated cAMP production. Although such correspondence may be coincidental, the uniformity of the finding strongly suggests that it is representative of the quantitative relationship between these two events under physiologic conditions. As expected, the peptide [^8Nle , ^{18}Nle , ^{34}Tyr]bPTH(3-34)amide, a known competitive inhibitor of PTH in vitro (14, 15), was found to competitively inhibit the binding of [^{125}I]bPTH(1-34), to inhibit the stimulation of cAMP production, and to have no stimulatory effect on cAMP production in calvariae. These observations provide further evidence in favor of the specificity of the PTH binding we have observed.

The present investigation represents the initiation of what appears to be an extremely fertile ground for the study of the structure-function relationships of PTH hormone action in general and of the initial events in PTH action on bone cells in particular. In the future, it is important that the relative abilities of cultured isolated and functionally different bone cell populations to bind PTH be thoroughly investigated so that our observations, with respect to whole calvariae, that the osteoblastlike cell is the major direct osseous target of PTH can be buttressed or refuted. On the other hand, further exploration of the calvarial system described in this work will be helpful in approaching questions which can be better answered by using intact bone.

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