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Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: A single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium

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ABSTRACT **Parathyroid hormone (PTH), a major regulator of mineral ion metabolism, and PTH-related peptide (PTHrP), which causes hypercalcemia in some cancer patients, stimulate multiple signals (cAMP, inositol phosphates, and calcium) probably by activating common receptors in bone and kidney. Using expression cloning, we have isolated a cDNA clone encoding rat bone PTH/PTHrP receptor from rat osteosarcoma (ROS 17/2.8) cells. The rat bone PTH/PTHrP receptor is 78% identical to the opossum kidney receptor; this identity indicates striking conservation of this receptor across distant mammalian species. Additionally, the rat bone PTH/PTHrP receptor has significant homology to the secretin and calcitonin receptors but not to any other G protein-linked receptor. When expressed in COS cells, a single cDNA clone, expressing either rat bone or opossum kidney PTH/PTHrP receptor, mediates PTH and PTHrP stimulation of both adenylate cyclase and phospholipase C. These properties could explain the diversity of PTH action without the need to postulate other receptor subtypes.**

Parathyroid hormone (PTH) regulates mineral ion metabolism and bone turnover by activating specific receptors located on osteoblastic and renal tubular cells (1). In these tissues PTH stimulates multiple intracellular signals that include cAMP (2–6), inositol phosphate, and calcium (7–13) and activates both protein kinases A (14) and C (15). PTH-related peptide (PTHrP), which has partial homology to PTH (16), causes the syndrome of humoral hypercalcemia of malignancy, probably by activating receptors it shares with PTH (17–21). The diversity of PTH action in bone and kidney and the multiple signals stimulated by PTH have led to the hypothesis of multiple PTH receptor subtypes (1, 7–15). Here we report the expression cloning and the functional properties of a rat bone PTH/PTHrP receptor cDNA cloned from a ROS 17/2.8 cDNA library. Activation of this single receptor by PTH or PTHrP stimulates both adenylate cyclase and phospholipase C.

MATERIALS AND METHODS

Expression Cloning. A size-selected oligo(dT)-primed cDNA library (10^7 independent transformants) was constructed in pcDNA1 (Invitrogen, San Diego) from ROS 17/2.8 poly(A)$^+$ RNA using the method of Aruffo and Seed (22). Plasmid DNAs derived from 20 pools, each representing 10,000 independent *Escherichia coli* colonies, were expressed in COS-7 cells and screened by photoemulsion autoradiography (23). A positive pool was found that, when subdivided, yielded a single positive clone, R15B. Clone R15B was sequenced with the dideoxynucleotide chain-termination method (Sequenase, United States Biochemical).¶

Radioreceptor Assay. COS-7 cells transfected with the cDNA clone were plated in 24-well plates. The cells were incubated with ^{125}I-labeled [Nle^{8,18},Tyr^{34}]bovinePTH(1–34)NH$_2$ [PTH(1–34)] or ^{125}I-labeled [Tyr^{36}]PTHrP(1–36)NH$_2$ [PTHrP(1–36)] (100,000 cpm per well/0.5 ml) in the presence or absence of competing ligands at 15°C for 4 hr, using a Tris-based binding buffer (50 mM Tris·HCl, pH 7.7/100 mM NaCl/5 mM KCl/2 mM $CaCl_2$/5% heat-inactivated horse serum/0.05% heat-inactivated fetal bovine serum as described (17, 18). The competing ligands included PTH(1–34), PTHrP(1–36), [Nle^{8,18},Tyr^{34}]bovinePTH(3–34)NH$_2$ [PTH(3–34)], and [Nle^{8,18},Tyr^{34}]bovinePTH(7–34)NH$_2$ [PTH(7–34)]. Techniques used for radioiodination of PTH and PTHrP analogs were reported (17, 18).

Adenylate Cyclase and cAMP Stimulation Assays. cAMP accumulation was assayed in intact cells after incubating the cells with PTH(1–34) or PTHrP(1–36) in the presence of 2 mM isobutylmethylxanthine for 15 min at 37°C. Intracellular cAMP was extracted with 50 mM HCl and assayed by a specific radioimmunoassay as described (24). Adenylate cyclase activity was measured in membranes prepared from COS-7 cells expressing PTH/PTHrP receptors (24). The assay solution contains 25 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM ATP, 1 mM phosphocreatine, and 1 unit of creatinine kinase. The membranes were incubated with PTH(1–34) at 30°C for the indicated time periods with or without 10 μM GTP; cAMP was measured by radioimmunoassay (24).

Measurement of Intracellular Free Calcium ($[Ca^{2+}]_i$). Forty-eight hours after transfection, subconfluent COS-7 cells on glass coverslips were loaded with fura-2 by incubating them with the acetoxymethyl ester of fura-2 (fura-2 AM) (2 μM) for 30 min at 37°C. Fluorescence of two to five cells was measured with a Zeiss inverted microscope (emission light >450 nm) with cells excited at 350 and 380 nm by using a dual-wavelength excitation source (Photon Technology International, Princeton), as described (25). Calibration at the end of each experiment with 10 μM ionomycin was followed

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Abbreviations: PTH, parathyroid hormone; PTHrP, PTH-related peptide; InsP_3, inositol 1,4,5-trisphosphate plus inositol 1,3,4-trisphosphate; $[Ca^{2+}]_i$, intracellular free calcium.

¶The nucleotide sequence of clone R15B has been deposited in the GenBank data base (accession no. M77184).

by 10 mM EGTA, pH 8.3. $[Ca^{2+}]_i$ was calculated using standard equations, assuming a K_d of 224 nM.

Measurement of Inositol Phosphates. Thirty hours after transfection, subconfluent COS-7 cells in 10-cm plates were washed with phosphate-buffered saline and placed in inositol-free RPMI 1640 medium supplemented with 1% fetal calf serum and [^{3}H]myoinositol at 3 μCi/ml (1 Ci = 37 GBq), for 14 hr. Eight minutes before stimulation, 5 mM lithium chloride was added. After activation with the agonists for 60 sec, 10% (wt/vol) trichloroacetic acid was added. Freon amine was then added to the non-trichloroacetic acid-precipitable supernatant. The aqueous phase was removed and loaded onto a Partisil SAX-10 column (Whatman). Inositol phosphates were separated by using HPLC as described (26), and radioactivity was monitored with an on-line radioactivity detector (Berthold Analytical, Nashua, NH).

Southern and Northern (RNA) Blot Analysis. Total RNA (20 μg) was analyzed on formaldehyde/agarose gel and transferred onto nitrocellulose filters. Genomic DNA was digested with restriction enzymes, analyzed on agarose gel, denatured, and transferred onto nitrocellulose filters. The filters were hybridized with randomly labeled cDNA probes at 42°C for 12 hr and washed twice with 0.5× standard saline citrate/0.1% SDS at 65°C for 1 hr.

RESULTS

After the screening of 20 pools of the library, representing ≈200,000 independent clones, a positive pool was found.

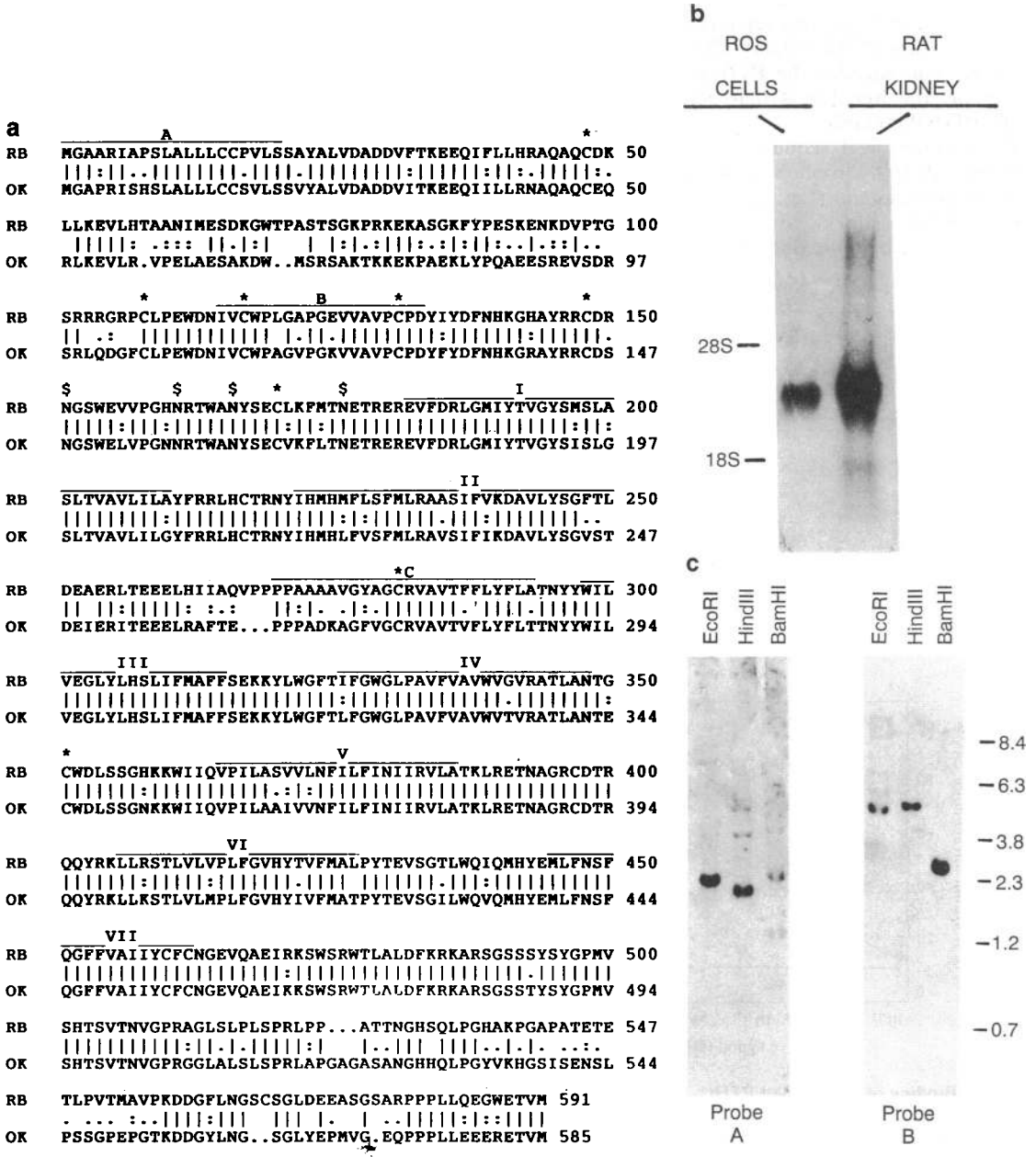

FIG. 1. (*a*) Analysis of amino acid sequence similarity between rat bone (RB) and opossum kidney (OK) PTH receptors with the GAP program of the Genetics Computer Group (Madison, WI). Hydrophobic domains are labeled A, B, C, and I–VII. Predicted extracellular cysteine residues (*) and potential extracellular glycosylation sites ($) are indicated. Lines (|) between residues refer to identity; dots (: and .) refer to high and low degree of amino acid homology, respectively. (*b*) Northern blot analysis of total RNA prepared from ROS 17/2.8 cells and from rat kidney. A total of 20 μg of RNA was loaded per lane. The blot was hybridized to a cDNA probe spanning nucleotides 1–1810. Positions of 28S and 18S ribosomal RNAs are shown. (*c*) Southern blot analysis of rat genomic DNA digested with *Hin*dIII, *Eco*RI, and *Bam*HI. The blot was hybridized with a cDNA fragment spanning nucleotides 1–1234 (probe A) or nucleotides 1235–1810 (probe B).

This pool was subdivided until a single clone was isolated. The cDNA clone R15B contains an open reading frame between nucleotides 73 and 1845 that encodes a 591-amino acid protein (Fig. 1*a*). The deduced amino acid sequence of the PTH/PTHrP receptor reveals significant homology to the rat secretin receptor (42% identity and 62% similarity) (27) and to the porcine calcitonin receptor (30% identity and 51% similarity) (28). The rat bone and the opossum kidney (29) PTH/PTHrP receptors are strikingly homologous; 77.8% of the amino acids are identical (Fig. 1*a*). This sequence conservation and the single mRNA species found on Northern blots of RNA from both rat osteosarcoma cells and rat kidney tissues (Fig. 1*b*) indicate that the PTH/PTHrP receptor in these target tissues is probably the same. Southern blot analyses of rat genomic DNA show one major band with each digest (Fig. 1*c*). The minor bands seen in several lanes may well reflect cleavage within the multiple intervening sequences already found in the coding region of the rat PTH/PTHrP receptor gene (unpublished data). These data suggest that a single-copy gene encodes the PTH/PTHrP receptor and further support the hypothesis that bone and kidney share one PTH/PTHrP receptor.

Analysis of the amino acid sequence by the method of Klein *et al.* (30) reveals 10 hydrophobic domains of sufficient length to be membrane spanning (Fig. 1*a*). These domains are similar to those of the opossum kidney PTH/PTHrP receptor (29). Assignment of hydrophobic domains A, B, and C and transmembrane domains I–VII (Fig. 1*a*) was based on the assumption that G protein-linked receptors contain seven transmembrane domains and on the significant sequence homology with the rat secretin and porcine calcitonin receptors; both lack hydrophobic domains B and C. Thus, hydrophobic domain A is probably a signal peptide; hydrophobic domain B is likely to be extracellular because it occurs before four potential N-linked glycosylation sites that must be extracellular; hydrophobic domain C is thought to be part of the first extracellular loop; and hydrophobic domains I–VII probably span the membrane. However, the exact topographical organization of the PTH/PTHrP receptor within the membrane will require experimental verification.

Radioiodinated analogs of both PTH and PTHrP, PTH(1–34) and PTHrP(1–36), specifically bound to the receptor protein encoded by clone R15B (Fig. 2*A* and *B*). The binding affinities of PTH(1–34) and PTHrP(1–36) were equivalent (apparent K_d values, 10 nM), when either ^{125}I-labeled PTH or PTHrP analog was used as radioligand (Fig. 2*A* and *B*). Other PTH analogs, PTH(3–34) and PTH(7–34), competed for binding with K_d values of 5×10^{-8} and 10^{-5} M, respectively. This order of binding affinity for PTH analogs was the same as seen in native ROS 17/2.8 cells. These data confirm that R15B encodes a receptor protein that binds both PTH and PTHrP analogs with equivalent affinity.

Because PTH and PTHrP both activate adenylate cyclase in target cells (2–6), we examined PTH- and PTHrP-

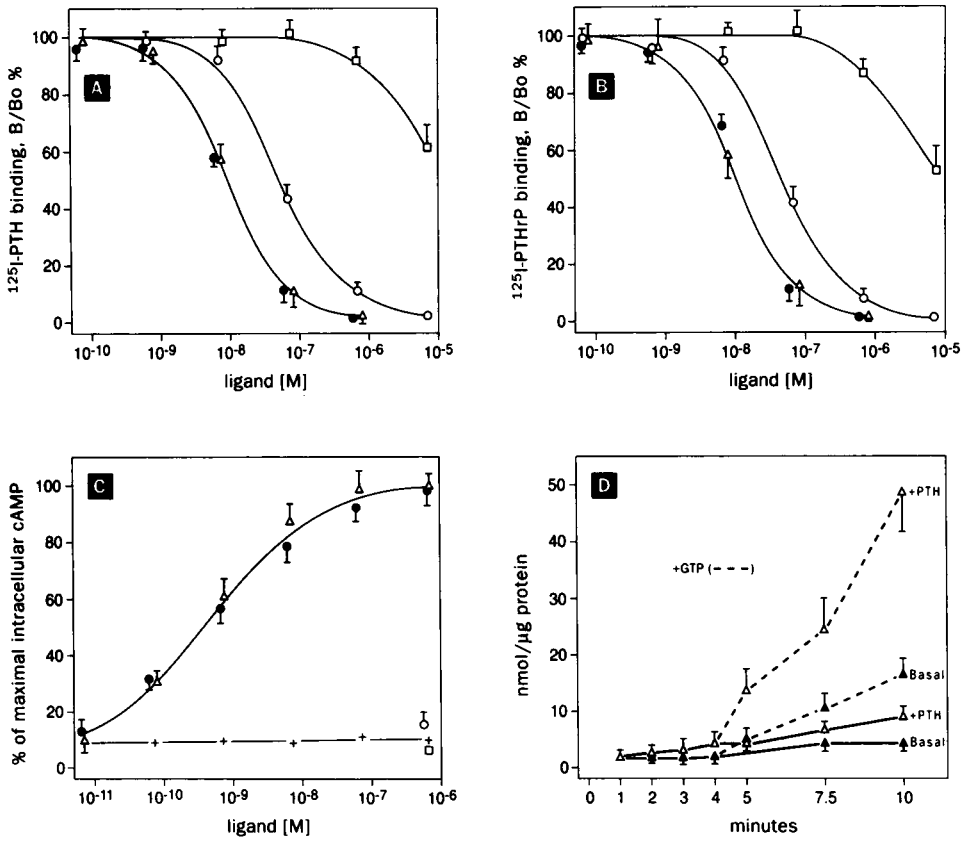

FIG. 2. (*A* and *B*) Binding of ^{125}I-labeled PTH(1–34) (*A*) and ^{125}I-labeled PTHrP(1–36) (*B*) to COS-7 cells expressing rat bone PTH receptor; competition was with PTHrP(1–36) (●), PTH(1–34) (△), PTH(3–34) (○), and PTH(7–34) (□). COS-7 cells expressing R15B were incubated with the radioligand (200,000 cpm per well) in the presence of increased concentrations of competing ligands. *B*/*Bo* (bound/bound in absence of competitor) (means ± SD; $n = 3$) was computed and plotted. Using photoemulsion autoradiography, we estimated that 10–30% of the cells expressed receptor cDNA. Therefore, the number of expressed receptors per cell was calculated at 800,000–1,200,000. (*C*) Stimulation of intracellular cAMP accumulation (37°C, 15 min, 2 mM isobutylmethylxanthine) by PTHrP(1–36) (●), PTH(1–34) (△), PTH(3–34) (○), and PTH(7–34) (□) in COS-7 cells expressing rat bone receptor. Data are means ± SD ($n = 3$). Basal values were 8 ± 3 pmol per 15 min per well, and maximal stimulated values were 125 ± 8 pmol per 15 min per well. (*D*) PTH stimulation of adenylate cyclase activity in membrane fractions prepared from COS-7 cells expressing R15B. Membranes were incubated with or without 100 nM of PTH(1–34) for 1–10 min at 30°C without GTP (—); GTP (10 μM) was added after 4 min of incubation (- - -). Data are means ± SD of triplicate samples (each sample was assayed for adenylate cyclase activity in duplicates).

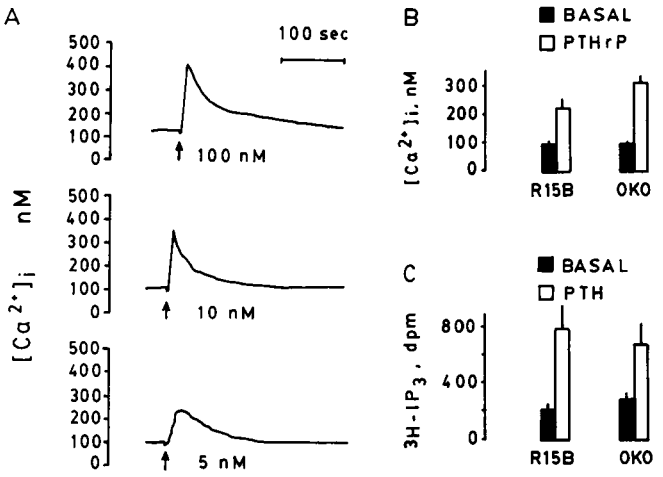

FIG. 3. $[Ca^{2+}]_i$ transient and total InsP_3 production stimulated by either PTH(1–34) or PTHrP(1–36) in COS-7 cells expressing either R15B or OKO. (*A*) Effects of PTH(1–34) (5, 10, and 100 nM) on $[Ca^{2+}]_i$ in COS-7 cells expressing R15B. Arrows indicate time of PTH(1–34) addition. (*B*) Effects of PTHrP(1–36) (100 nM) on $[Ca^{2+}]_i$ in COS-7 cells expressing either R15B or OKO, as indicated. Mean ± SD of peak increase in $[Ca^{2+}]_i$ was calculated from four to seven independent experiments. Levels of stimulated $[Ca^{2+}]_i$ in cells expressing R15B were not statistically different from levels in cells expressing OKO. (*C*) Stimulation of total InsP_3 production (60 sec, 37°C, 5 mM LiCl) by PTH(1–34) (100 nM) in COS-7 cells expressing either R15B or OKO, as indicated. In similar experiments, PTHrP(1–36) (100 nM) increased total InsP_3 production from 198 to 536 dpm per plate and from 349 to 899 dpm per plate in COS-7 cells expressing either OKO or R15B, respectively.

stimulated cAMP responses of the expressed receptors. Both PTH(1–34) and PTHrP(1–36) stimulated cAMP accumulation (8- to 14-fold) in COS-7 cells expressing R15B (Fig. 2*C*), but neither peptide affected cAMP accumulation in COS-7 cells transfected with the vector alone. The ED_{50} for both ligands was 0.5 nM, similar to that seen in ROS 17/2.8 cells (Fig. 2*C*). Furthermore, in membranes prepared from COS-7 cells expressing R15B, PTH(1–34) and PTHrP(1–36) stimulated adenylate cyclase in a GTP-dependent manner (Fig. 2*D*); these data demonstrate coupling of the PTH/PTHrP receptor to adenylate cyclase through a G protein, presumably G_s.

In addition to stimulating adenylate cyclase, PTH increases $[Ca^{2+}]_i$ in target cells (11). Therefore, we determined the effects of PTH(1–34) and PTHrP(1–36) on $[Ca^{2+}]_i$ in COS-7 cells expressing R15B. As controls, we used untransfected COS-7 cells and COS-7 cells transfected with either vector alone or human β_2-adrenergic receptor cDNA (31). Addition of PTH(1–34) in doses as low as 5 nM (lowest dose tested) increased $[Ca^{2+}]_i$ in COS-7 cells transfected with R15B (Fig. 3*A*). Similar data were obtained when PTHrP(1–36) was used, rather than PTH(1–34) (data not shown).

Binding affinities of PTH(1–34) and PTHrP(1–36) and their V_{max} and ED_{50} for stimulation of cAMP accumulation in COS-7 cells expressing the opossum kidney PTH/PTHrP receptor cDNA, OKO (29), are similar to those obtained in COS-7 cells expressing R15B (data not shown). Therefore, we tested whether OKO also increases $[Ca^{2+}]_i$ when activated by PTH(1–34) and PTHrP(1–36). Addition of PTHrP(1–36) (100 nM) to COS-7 cells expressing either R15B or OKO, resulted in equivalent increases in $[Ca^{2+}]_i$ (Fig. 3*B*). Similar results were obtained when PTH(1–34) (100 nM) was used, rather than PTHrP(1–36) (data not shown). The rapid increase in $[Ca^{2+}]_i$ induced by PTH(1–34) or PTHrP(1–36) in COS-7 cells expressing R15B and OKO was associated with activation of phospholipase C, as evidenced by an increase in total inositol-1,4,5 plus 1,3,4 trisphosphate (InsP_3) production (Fig. 3*C*). No increase in $[Ca^{2+}]_i$ or total InsP_3 production was seen in any controls after challenge with PTH and PTHrP analogs, or after treatment with the β-adrenergic agonist isoproterenol.

DISCUSSION

In this report we describe the isolation of a cDNA clone from rat osteoblast-like cells by using an expression cloning method. The high degree of sequence identity between PTH/PTHrP receptors in eutherian and marsupial mammals indicates that the PTH/PTHrP receptor has been highly conserved during evolution of mammalian species. Their sequence conservation was not a simple consequence of the manner of their isolation because each receptor was cloned independently by a method based on ligand binding rather than on nucleotide sequence hybridization. The regions that are least homologous in the two receptors are located in the amino-terminal extracellular extension (amino acids 57–109), the first extracellular loop (amino acids 262–279), and the carboxyl-terminal tail (amino acids 535–556). These regions may confer different biological properties to these two receptors. However, our data indicate that both of these receptors bind PTH(1–34) and PTHrP(1–36) equivalently, and, when activated, both stimulate adenylate cyclase and phospholipase C efficiently.

It should be noted that activation of the human β_2-adrenergic receptor, expressed in COS-7 cells, did not stimulate phospholipase C activity and did not increase $[Ca^{2+}]_i$. Because isoproterenol dramatically increases cAMP accumulation in COS-7 cells expressing the β_2-adrenergic receptor (data not shown) but does not raise either $[Ca^{2+}]_i$ or InsP_3 levels, we can conclude that the rise of $[Ca^{2+}]_i$ and InsP_3 after PTH treatment is not simply a consequence of activation of adenylate cyclase. Additionally, the GTP-dependent stimulation of adenylate cyclase by PTH in broken cell membrane preparations shows direct coupling of this receptor to G_s and indicates that stimulation of adenylate cyclase is not secondary to activation of other intracellular second-messenger systems of the intact cell.

Nanomolar concentrations of PTH are known to increase cAMP, $[Ca^{2+}]_i$, and InsP_3 in target cells (2–13). Our data, which show that these second messengers also are increased when a single expressed PTH/PTHrP receptor is activated by similar doses of the hormone, indicate that the same receptor efficiently couples to multiple effector systems. Recently, a thyrotropin receptor has been shown to have similar properties of coupling efficiently to G_s and a G protein(s) linked to phospholipase C (32); no other examples of this pattern have been reported. The structural features of these receptors responsible for these common functional properties remain to be defined. Conservation of this multiple signaling capacity is particularly interesting because PTH/PTHrP and thyrotropin receptors appear only distantly related and share <10% sequence homology.

PTH activation of both adenylate cyclase and phospholipase C in multiple tissues has suggested that multiple receptors mediate these activities (1). The striking sequence homology between the rat bone and the opossum kidney receptors, the data from hybridization studies, and the finding that the activated receptors efficiently stimulate multiple effectors suggest that only a single receptor mediates all the actions of PTH. Other receptors for PTH may well exist in these and other tissues; we have not yet systematically searched for such receptors. The many receptors expressed in COS-7 cells makes it difficult to be sure how completely stimulation of two signaling pathways by cloned receptors in COS-7 cells can quantitatively explain the stimulation of these signaling pathways in normal bone cells. The cloned receptors described here, however, stimulate intracellular accumulation of both cAMP and total InsP_3 efficiently. This

striking versatility of the receptor makes it unnecessary to postulate multiple PTH receptors.

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