Synthetic Peptides Comprising the Amino-terminal Sequence of a Parathyroid Hormone-like Protein from Human Malignancies

BINDING TO PARATHYROID HORMONE RECEPTORS AND ACTIVATION OF ADENYLATE CYCLASE IN BONE CELLS AND KIDNEY*

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A tumor-derived protein with a spectrum of biologic activities remarkably similar to that of parathyroid hormone (PTH) has recently been purified and its sequence deduced from cloned cDNA. This PTH-like protein (PLP) has substantial sequence homology with PTH only in the amino-terminal 1–13 region and shows little similarity to other regions of PTH thought to be important for binding to receptors. In the present study, we compared the actions of two synthetic PLP peptides, PLP-(1-34)amide and [Tyr36]PLP-(1–36)amide, with those of bovine parathyroid hormone (bPTH)-(1–34) on receptors and adenylate cyclase in bone cells and in renal membranes. Synthetic PLP peptides were potent activators of adenylate cyclase in canine renal membranes (EC50 = 3.0 nm) and in UMR-106 osteosarcoma cells (EC50 = 0.05 nm). Bovine PTH-(1–34) was 6-fold more potent than the PLP peptides in renal membranes, but was 2-fold less potent in UMR-106 cells. A competitive PTH receptor antagonist, [Tyr36]bPTH-(7–34)amide, rapidly and fully inhibited adenylate cyclase stimulation by the PLP peptides as well as bPTH-(1–34). Competitive binding experiments with 125I-labeled PLP peptides revealed the presence of high affinity PLP receptors in UMR-106 cells IC50 = 3–4 nm) and in renal membranes (IC50 = 0.3 nm). There was no evidence of heterogeneity of PLP receptors. Bovine PTH-(1–34) was equipotent with the PLP peptides in binding to PLP receptors. Likewise, PLP peptides and bPTH-(1–34) were equipotent in competing with 125I-bPTH-(1–34) for binding to PTH receptors in renal membranes. Photoaffinity cross-linking experiments revealed that PTH and PLP peptides both interact with a major 85-kDa and minor 55- and 130-kDa components of canine renal membranes. We conclude that PTH and PLP activate adenylate cyclase by binding to common receptors in bone and kidney. The results further imply that subtle differences exist between PTH and PLP peptides in their ability to induce receptor-adenylate cyclase coupling.

A wide variety of human and animal tumors produce a protein(s) with a spectrum of biologic activities resembling that of parathyroid hormone (PTH). Such PTH-like proteins (PLPs) have been purified to homogeneity from extracts of breast (1) and lung (2) carcinoma, and from medium conditioned by lung (3) and renal (4) carcinoma cell lines. These proteins are immunologically distinguishable from PTH, yet reproduce the major biochemical and physiologic effects of PTH in model in vitro systems, including activation of adenylate cyclase in bone and kidney (1–8), stimulation of resorption of fetal rat limb bones (4, 9–12), and inhibition of sodium-dependent phosphate uptake in opossum kidney cells (4, 13).

Recently, the amino-terminal sequences of PLPs from four sources have been reported (2–4). A single consensus sequence was obtained in which 8 of the amino-terminal 13 residues are identical to those present in human PTH. Based upon its cDNA sequence, PLP derived from lung carcinoma cells (14) and from a renal carcinoma (15) was found to be a 141-amino acid protein (compared to the 84-amino acid PTH) with significant sequence homology to PTH only in this amino-terminal 1–13 region. The primary PLP transcript appears to undergo alternative splicing in renal carcinoma cell line 786-O which expresses an additional PLP mRNA encoding an identical protein truncated by two amino acids at the carboxyl terminus (16). Sequence homology between PTH and PLP does not extend to the 25–34 positions thought to be critical for PTH binding to receptors (17).

Nonetheless, available evidence supports the notion that the PTH-like effects of PLP occur as a result of its interaction with adenylate cyclase-coupled PTH receptors in bone and kidney. Thus, competitive antagonists of PTH block PLP-induced activation of adenylate cyclase in bone and kidney (18, 19). Furthermore, the results of competitive binding experiments with 125I-labeled PLP demonstrated competition between PTH and PLP for receptors in canine renal membranes (4).

In the present study, we evaluated the biologic activities of synthetic PLP peptides in binding to receptors and activating adenylate cyclase in canine renal plasma membranes and in rat osteoblast-like osteosarcoma cells. The results indicate that, as with PTH, the biologic activity is largely contained within the protein’s amino-terminal 1–34 sequence. Furthermore, radioligand binding and photoaffinity cross-linking

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1 The abbreviations used are: PTH, parathyroid hormone; PLP, parathyroid hormone-like protein; bPTH, bovine parathyroid hormone; SDS, sodium dodecyl sulfate; G, stimulatory GTP-binding component of adenylate cyclase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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studies indicate that amino-terminal PLP peptides and PTH-(1–34) interact with a single adenylyl cyclase-coupled PTH receptor in these systems.

EXPERIMENTAL PROCEDURES

Materials—Synthetic bPTH-(1–34) (5000 units/mg) was purchased from Bachem, Inc. (Torrance, CA). Synthetic PLP peptides were synthesized according to the amino acid sequence deduced by Suva et al. (14). The 1–34 peptide sequence of the PTH-like protein (PLP-(1–36)amide) was a generous gift of Dr. Michael Rosenblatt (Merck Sharp & Dohme Research Laboratories, West Point, PA). [Tyr3']bPTH-(1–36)amide was a generous gift of Dr. Andrew Stewart (West Haven, Veterans Administrations Medical Center, West Haven, CT). Synthetic [Tyr3']bPTH-(7–34)amide was purchased from Peninsula Laboratories (San Carlos, CA). Clonal rat osteosarcoma cell line UMR-106 was kindly provided by Dr. T. John Martin (Heidelberg, Australia).

Radioiodination—Bovine PTH-(1–34) was iodinated by an electrolytic procedure to a specific activity of 100–200 μCi/μg, as described previously (20). PLP-(1–34)amide and [Tyr3']PLP-(1–36)amide were iodinated by a modification of the chloramine-T procedure of Hunter and Greenwood (21). Briefly, 10 μg of PLP peptide was added to 1–2 mCi of Na125I in 0.1 ml of 0.05 M sodium phosphate, pH 7.5. Iodination was allowed to proceed for 3 min following the addition of 0.6 μg of chloramine T. Fresh chlormine T (0.5 μg) was added every 60 s. The reaction was terminated by the addition of 20 μl of 1.0 mM N-mercaptoethanol, 20 μl of 11 mg/ml N-acetyltosynine, and 200 μl of 10 mg/ml potassium iodide in phosphate buffer. The specific activity of the PLP peptides ranged from 70 to 100 μCi/μg. 125I-labeled PTH and PLP peptides were purified by chromatography over Sephadex LH-20 followed by reverse-phase high performance liquid chromatography over a C-18 column, as described previously for [35S-bPTH-(1–34) (22).

Binding of [125I-bPTH-(1–34) and [125I-labeled peptides to canine renal plasma membranes was assessed as described previously for [125I-bPTH-(1–34) (23). Labeled ligands were present at a concentration of about 106 cpm/100 μl incubation volume. Initial experiments indicated that steady-state binding of ligands occurred after a 120-min incubation at 30 °C, and these conditions were used in all subsequent experiments. Binding of labeled ligands to UMR-106 cells was assessed as follows: UMR-106 cells were grown to confluence in 24-well cluster plates, as described previously (24). Confluent cells were incubated at 37 °C in serum-free minimal essential medium-Earle’s balanced salt solution for at least 1 h and then were exposed to 150 μl of minimal essential medium-Earle’s balanced salt solution containing 20 mM Hepes, 1–2 × 106 cpm of labeled PTH or PLP, and unlabeled peptides as indicated. Incubations were carried out at room temperature for 1 h, at which time steady-state binding was achieved. Following aspiration of the medium, the cells were washed three times with 1.0 ml of ice-cold phosphate-buffered saline and were then scraped from the dishes in 0.8 M NaOH for γ-counting.

Adenylate Cyclase—Adenylate cyclase activity in canine renal plasma membranes was assessed by the conversion of [32P]ATP to [32P]cyclic AMP, as described previously (25). Unless otherwise indicated, assays were carried out in the presence of 0.1 mM GTP. Adenylate cyclase activity in UMR-106 cells was assessed as the production of cyclic AMP during a 10-min incubation at room temperature in the presence of 1.0 mM isobutylmethylxanthine and 50 nM forskolin, as described previously (24).

Photocross-linking—[125I-bPTH-(1–34) and [125I-PLP-(1–34)amide were covalently attached to binding sites in canine renal membranes using the heterobifunctional cross-linker HSAB, as described previously for the identification of PTH receptors (26). Membranes were subjected to SDS-polyacrylamide gel electrophoresis using gels polymerized in a 5–20% gradient of acrylamide.

RESULTS

Synthetic PLP peptides were found to be potent activators of adenylyl cyclase activity in canine renal plasma membranes and in UMR-106 osteoblast-like osteosarcoma cells (Fig. 1). Renal membrane adenylate cyclase was activated approximately 20-fold by maximally effective concentrations of the PLP peptides and by bPTH-(1–34). In this system, PLP-(1–34)amide was equipotent with [Tyr3']bPTH-(1–36)amide (EC50 values of 3.0 nM); the PLP peptides were about 6-fold less potent than bPTH-(1–34) (EC50 value of 0.5 nM). In contrast, the PLP peptides were slightly more potent than bPTH-(1–34) in stimulating adenylate cyclase in UMR-106 rat osteosarcoma cells. Values shown are the mean ± S.E. of triplicate determinations. Results are representative of those obtained in four similar experiments.

The kinetics of renal adenylate cyclase activity in the presence of both bPTH-(1–34) and PLP-(1–34)amide were linear over at least the 30-min time course of the standard assay (Fig. 2). Inclusion of an ~100-fold molar excess of a competitive antagonist of PTH action, [Tyr4']bPTH-(7–34)amide, fully blocked adenylate cyclase stimulation by PLP-(1–34)amide as well as bPTH-(1–34). Once activated by bPTH-(1–34) or by PLP-(1–34)amide, adenylate cyclase activity was inhibited to basal levels within minutes after the
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FIG. 2. Kinetics of renal membrane adenylate cyclase activation by bPTH-(1-34) (●) and PLP-(1-34)amide (○), and inhibition by [Tyr36]bPTH-(7-34)amide. At time 0, 100 ng/ml of bPTH-(1-34) or PLP-(1-34)amide were added either in the absence (solid lines) or presence (dashed lines) of 10 pg/ml [Tyr36]bPTH-(7-34)amide. Incubations proceeded for 15 min, at which time tubes containing bPTH-(1-34) and PLP-(1-34)amide received either vehicle (solid lines) or 10 pg/ml of [Tyr36]bPTH-(7-34)amide (dashed lines). Adenylate cyclase is expressed as nanomoles of cAMP/mg protein.

addition of [Tyr36]bPTH-(7-34)amide.

The inhibition of PLP-stimulated adenylate cyclase by the competitive PTH angatonist [Tyr36]bPTH-(7-34)amide indicates that PLP may act via the PTH receptor. This was confirmed directly in competitive binding experiments using 125I-bPTH-(1-34) (Fig. 3). PLP-(1-34)amide competed with 125I-bPTH-(1-34) for renal PTH receptors with a potency (IC50 = 0.3 nM) similar to that of bPTH-(1-34) (IC50 = 0.3 nM). [Tyr36]PLP-(1-36)amide was equipotent with PLP-(1-34)amide in binding to canine renal PTH receptors (not shown).

Specific PLP binding sites were identified directly in canine renal membranes using 125I-[Tyr36]PLP-(1-36)amide (Fig. 4). 125I-PLP peptides consistently produced greater specific binding than 125I-bPTH-(1-34), probably due to the greater stability of PLP (which lacks methionine) under the oxidizing conditions associated with iodination. In this experiment, approximately 30% of the added 125I-[Tyr36]PLP-(1-36)amide was specifically bound; nonspecific binding was <2%. Unlabeled [Tyr36]PLP-(1-34)amide bound to PLP receptors with an IC50 of 0.3 nM. bPTH-(1-34) bound to PLP receptors with an affinity identical to that of [Tyr36]PLP-(1-34)amide, and, at 20 nM, fully saturated PLP receptors. Scatchard analysis of these data indicates the presence of high affinity PLP receptors (Kd = 0.28 nM; Bmax = 0.53 pmol/mg protein). No evidence was obtained for heterogeneity of renal PLP receptors.

In UMR-106 osteosarcoma cells, specific PLP binding sites were identified using 125I-[Tyr36]PLP-(1-34)amide (Fig. 5). Bovine PTH-(1-34) and PLP-(1-34)amide were approximately equipotent in binding to PLP receptors in these cells (EC50 = 3-4 nM). At a concentration of 0.2 μM, bPTH-(1-34) saturated PLP receptors.

The GTP binding protein G, interacts with the PTH receptor during hormonal activation of adenylate cyclase (27). This interaction is reflected in a marked increase in the sensitivity of adenylate cyclase to PTH induced by stable analogs of GTP (28), and in an enhancement by GTP (or analogs) of the rate of dissociation of PTH from its receptor. Gpp(NH)p markedly accelerated the rates of dissociation of 125I-bPTH-(1-34) and 125I-PLP-(1-34)amide from binding sites in canine...
minor species are more evident at longer exposures of the autoradiogram. Unlabeled bPTH-(1-34) and PLP-(1-34)amide, at 1.0 µg/ml, fully inhibited labeling of these components. 125I-PLP-(1-34)amide produced labeling of membrane components having molecular weights identical to those of membrane components labeled with 125I-bPTH-(1-34). Additional lower molecular weight species were evident, as well. Unlabeled bPTH-(1-34) and unlabeled PLP-(1-34)amide fully inhibited labeling of the major 85-kDa and minor 130-kDa forms, and markedly reduced labeling of the 55-kDa form.

DISCUSSION

The results of the present study establish that PLP activates adenylate cyclase in bone and kidney by interacting with membrane receptors previously thought to be specific for parathyroid hormone. In support of this conclusion, synthetic PLP peptides competed with 125I-bPTH-(1-34) for specific binding sites in canine renal plasma membranes. Similar results have recently been reported by Horiuchi et al. (29). Furthermore, synthetic 125I-PLP peptides interacted with specific binding sites in UMR-106 osteoblast-like osteosarcoma cells and in renal membranes; bPTH-(1-34) displayed an affinity for these sites indistinguishable from its affinity for PTH receptors. Photoaffinity cross-linking of 125I-PLP-(1-34)amide to renal membranes revealed a major receptor species of 85 kDa and minor forms of 55 and 130 kDa on SDS-polyacrylamide gel electrophoresis. Renal membrane proteins of identical apparent molecular weight have been shown to be components of the adenylate cyclase-coupled PTH receptor (26). The ability of synthetic amino-terminal PLP peptides to bind to PTH receptors is consonant with recent reports that these peptides reproduce all of the major physiologic actions of PTH on bone and kidney (29-34).

The present results also demonstrate that PLP peptides bind to PTH receptors in canine renal membranes and in UMR-106 cells with an affinity virtually identical to that of bPTH-(1-34). This is a remarkable finding, given the lack of sequence homology between PLP and PTH in the domain of PTH thought to be essential for receptor binding. The results of extensive investigation of the structure-function relationships for PTH indicate that determinants in the 25-34 region of the peptide are critical for binding to receptors whereas the 1-7 region provides determinants that are required to elicit adenylate cyclase activation once binding occurs (17). A comparison of the primary structure of the 1-34 region of PTH with that of PLP (14-16) reveals that sequence homologies...
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are largely restricted to the 1–13 region of the peptides.  

bPTH: NH₂-AVSEIQFSTNLKHLSMVREWLRKKLQVHNF  

PLP: NH₂-AVSEHQLLHDKGSTQDRLRRFLHLIAEHTA  

Kemp et al. (30) have reported that, with respect to cAMP production in UMR-106-01 cells, PLP-(1–14) and PLP-(1–20) are inactive and PLP-(1–25) is <0.01% as active as PLP-(1–34). These results reveal marked similarities in the structure-function relationships for PLP and PTH, and in particular point to the 25–34 region of PLP as a critical determinant of its receptor binding. PTH and PLP share a single common amino acid in this region (His²⁶), while five additional positions (25, 26, 28, 30–32) have residues with conservative differences when assessed according to Dayhoff et al. (35). Apparentley, the mechanism by which this domain confers the ability to bind to PTH receptors permits extensive diversity in primary structure.

We have reported (4) that native, purified PLP is 10-20 times more potent (relative to bPTH-(1–34)) in stimulating adenylate cyclase in osteosarcoma cells than in renal membranes. This was apparently not due to differential degradation of bPTH-(1–34) and PLP under the assay conditions used (24). The results of the present study demonstrate that a similar potency difference obtains for synthetic PLP-(1–34)amide, confirming the recent report of Horiuchi et al. (29). The results rule out the possibility that this is explained by an enhanced affinity of bone cell PTH receptors for PLP versus bPTH-(1–34). It is possible that differential degradation of synthetic PTH and PLP peptides accounts for the potency difference. Alternatively, the peptides may differ quantitatively in their ability to induce receptor-adenylate cyclase coupling. It is of interest in this regard that whereas adenylate cyclase coupling induced by PTH and PLP also appear to interact with a common receptor in canine renal membranes, the PLP peptides were 6-fold less potent than bPTH-(1–34) in activating adenylate cyclase when occupied by PTH than by PLP peptides.

The kinetics of receptor binding and adenylate cyclase activation by PTH and PLP peptides in renal membranes were similar. Adenylate cyclase activity reached steady state within a minute after addition of either peptide, and enzyme activity returned to basal immediately following the addition of an excess of the competitive antagonist [Tyr²⁸]bPTH-(7–34)amide (Fig. 2). The kinetics of dissociation of ¹²⁵I-bPTH-(1–34) and of ¹²⁵I-PLP-(1–34)amide from renal receptors were also similar. The t₀ for dissociation of both peptides exceeded 120 min in the absence of guanyl nucleotides. Our previous results with ¹²⁵I-bPTH-(1–34) indicate that this slowly dissociating state represents a ternary PTH-receptor-Gₐ complex (25, 27). Consistent with this, the addition of Gpp(NH)p markedly increased the rate of dissociation of both labeled peptides (t₀ < 2 min). Gpp(NH)p also produced a 10-fold enhancement of the sensitivity of renal adenylate cyclase to both the PTH and PLP peptides. These results provide no evidence for gross differences in receptor-Gₐ adenylate cyclase coupling induced by PTH versus PLP peptides.

We have previously shown that ¹²⁵I-labeled native PLP (purified from human renal carcinoma cells) bound to canine renal membranes and that a portion of this binding was displaced by unlabeled bPTH-(1–34) (4). However, bPTH-(1–34) failed to displace 25% of tracer binding, raising the possibility that membranes might contain a specific PLP binding site in addition to PTH receptors. In the present study, the specific binding of ¹²⁵I-PLP-(1–34)amide was fully displaced by unlabeled bPTH-(1–34) both in renal membranes and in UMR-106 cells. Scatchard analysis demonstrated that the PLP/PTH receptor was the only class of specific PTH binding sites evident in canine renal membranes. Minor differences were evident in the pattern of renal membrane proteins labeled by ¹²⁵I-bPTH-(1–34). The former ligand produced relatively greater labeling of the 55-kDa components, as well as labeling several lower molecular weight species. The reason for these differences is unclear. However, in no instance was labeling inhibited by unlabeled PLP-(1–34)amide but not by unlabeled bPTH-(1–34). These results thus provide no evidence for the presence in bone or kidney of specific PLP receptors distinct from the PTH receptor. Further studies with the native 16-kDa PLP are needed to establish whether determinants in the 35–141 region of the peptide may mediate interactions of PLP with unique receptors.

In summary, synthetic PTH and PLP peptides activate adenylate cyclase in bone and kidney by interacting with common receptors. Synthetic PLP peptides are equipotent with bPTH-(1–34) in binding to these receptors, but, in renal membranes, occupancy of PLP peptides appears to induce coupling to adenylate cyclase that is less efficient than that induced by bPTH-(1–34). This difference notwithstanding, our results provide strong support for the concept that the PTH-like biologic effects of PLP derive from its activation of PTH receptors in bone and kidney.


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