Characterization of Canine Renal Receptors for the Parathyroid Hormone-like Protein Associated with Humoral Hypercalcemia of Malignancy*

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Parathyroid hormone-like proteins (PTHLP) display actions in the kidney which are similar to those of parathyroid hormone (PTH). We compared the binding properties of PTHLP and PTH in canine renal cortical membranes to determine if they interacted with the same or different receptors.

Radioiodination to high specific activity (greater than 400 Ci/mg) of [Nle8,Tyr34]human PTH-(1-34)amide and [Tyr38]PTHLP-(1-36)amide was performed using the lactoperoxidase method. Complete enzymatic digestion of both radioligands demonstrated that the peptides were monoidinated. Both radioligands retained full biological activity in the renal adenylate cyclase assay, and neither was significantly degraded during incubation with highly purified canine renal membranes under binding assays conditions.

Specific binding reached equilibrium by 20 min at 20 °C. Competition binding studies using unlabelled [Nle8,Tyr34]human PTH-(1-34)amide, [Tyr38]PTHLP-(1-36)amide, and bovine PTH-(1-34) with either radioligand revealed similar binding affinities for all three peptides. Biologically inactive PTHLP fragments did not show significant displacement. In contrast to its similar binding affinity, [Tyr38]PTHLP-(1-36)amide was 6-15-fold less potent than bovine PTH-(1-34) in the renal adenylate cyclase assay, suggesting less efficient receptor-effector coupling. Photoaffinity cross-linking using either radioligand in canine renal membrane labeled indistinguishable 70,000-dalton proteins. In the presence of multiple protease inhibitors, binding to an 85-kDa component was observed. Labeling of both receptor forms was specifically abolished by an excess of either cold peptide and dose-response curves using affinity cross-linked membranes corroborated the apparent binding affinities determined by conventional radioligand binding assays.

We conclude that PTHLP-(1-36) and amino-terminal PTH analogues bind to indistinguishable receptors in canine renal cortical membranes, but display differential coupling to post-receptor events.

Parathyroid hormone-like proteins (PTHLP) which appear to be responsible for many cases of humoral hypercalcemia of malignancy (HHM) have recently been isolated from several different human tumors, and a partial amino acid sequence has been determined (1-4). The full length amino acid sequence based on the complementary DNA sequence has also been elucidated (5-7). These peptides demonstrate marked amino-terminal (residues 1-13) homology to parathyroid hormone (PTH), with complete divergence thereafter. Marked amino-terminal (residues 1-13) homology to parathyroid hormone (PTH), with complete divergence thereafter. These peptides display actions similar to PTH in vivo and in vitro, including stimulation of bone resorption and promotion of renal cAMP and phosphorous excretion.

The purpose of the present study was to compare the properties of renal receptors for PTH and PTHLP and to determine if the two peptides interact with the same or different receptors. To accomplish this aim, we have used radioiodinated [Tyr38]PTHLP-(1-36)amide (PTHLP-(1-36)) and [Nle8,Tyr34]hPTH-(1-34)amide in competition binding studies using canine renal cortical membranes (CRCM). We have also examined the ability of these peptides to stimulate adenylate cyclase. Photoaffinity cross-linking of both radioligands to CRCM and analysis on SDS-polyacrylamide gels was also performed. The results indicate that PTHLP-(1-36) and amino-terminal PTH analogues appear to bind to the same or similar renal cortical receptors but that differences in signal transduction are evident.

MATERIALS AND METHODS AND RESULTS†

Competition Binding Studies—Inhibition of binding of [125I][Nle8,Tyr34]hPTH-(1-34)amide was performed using increasing concentrations of unlabeled [Nle8,Tyr34]hPTH-(1-34)amide, bPTH-(1-34), and PTHLP-(1-36) under equilib-

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1 The abbreviations used are: PTHLP, parathyroid hormone-like protein; PTH, parathyroid hormone; bPTH, bovine PTH; hPTH, human PTH; PTHLP-(1-36), [Tyr38]PTHLP-(1-36)amide; NNT, Nle8,Tyr34]hPTH-(1-34), [Nle8,Tyr34]human PTH-(1-34)amide; CRCM, canine renal cortical membranes; SDS, sodium dodecyl sulfate; HHM, humoral hypercalcemia of malignancy; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; Gpp(NH)p, guanylylimidodiphosphate; TFA, trifluoroacetic acid; HSAB, hydroxysuccinimidyldiazobenzene; NEM, N-ethylmaleimide; FMSF, phenylmethanesulfonyl fluoride; Me2SO, dimethyl sulfoxide.

2 Portions of this paper (including "Materials and Methods," part of "Results," Figs. 1-3, and Table 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
I.

**FIG. 4.** Competition binding studies of $^{125}$I-[Nle$^{18}$,Tyr$^{24}$]hPTH-(1-34)amide to canine renal membranes at 20 °C with unlabeled [Nle$^{18}$,Tyr$^{24}$]hPTH-(1-34) amide (A), bPTH-(1-34) (B), and [Tyr$^{24}$]PTHLP-(1-36)amide (C). Points represent the mean ± S.E. of triplicate determinations in three separate experiments (bPTH-(1-34) and [Tyr$^{24}$]PTHLP-(1-36)amide) or in two separate experiments ([Nle$^{18}$,Tyr$^{24}$]hPTH-(1-34)amide). Individual points were expressed as a percentage of the specific binding determined in the absence of unlabeled peptide (percentage of maximal specific binding). Inset indicates Scatchard analysis of a representative experiment. B/F, bound/free.

**TABLE II**

*In vitro activity of [Tyr$^{24}$]PTHLP-(1-36) amide compared to bPTH-(1-34)*

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_d/K_0$</th>
<th>$K_0$</th>
</tr>
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<tr>
<td>bPTH-(1-34)</td>
<td>$6.1 \pm 1.5$</td>
<td>$10.5 \pm 4.4$</td>
</tr>
<tr>
<td>[Nle$^{18}$,Tyr$^{24}$]hPTH-(1-34)amide</td>
<td>$11.5 \pm 2.5$</td>
<td>$14.0 \pm 5.4$</td>
</tr>
<tr>
<td>[Nle$^{18}$,Tyr$^{24}$]hPTH-(1-34)amide</td>
<td>$0.06 \pm 0.01$</td>
<td>$0.13 \pm 0.01$</td>
</tr>
</tbody>
</table>

$K_d$ values were determined by Scatchard analysis, and the $K_0$ values were derived from the $IC_50$ values as described previously (21). Adenylate cyclase stimulation was evaluated under standard assay conditions, employing partially purified canine renal membranes and 30-min incubations at 30 °C (19). Adenylate cyclase stimulation was also evaluated under binding assay conditions, using highly purified canine renal membranes in the presence of bacitracin (200 μg/ml) and 20-min incubations at 20 °C.

When $^{125}$I-PTHLP-(1-36) was used as the radioligand, all three synthetic peptides were approximately equipotent in inhibiting binding (Fig. 5). Binding dissociation constants for [Nle$^{18}$,Tyr$^{24}$]hPTH-(1-34)amide, bPTH-(1-34), and PTHLP-(1-36) were 8.5, 10.5, and 14.1 nM, respectively (Table 2). Both PTHLP-(49-74) and a synthetic 13-amino acid bio-inactive amino-terminal PTHLP (P$_1$ peptide) failed to inhibit binding of $^{125}$I-PTHLP-(1-36) to canine renal membranes (Fig. 5).

Representative Scatchard plots of the equilibrium binding data are presented in Figs. 4 and 5. The $B_{max}$ value for PTH analogue was 2.73 ± 0.31 pmol/mg protein and for PTHLP-(1-36) was 5.08 ± 0.56 pmol/mg protein. Analysis of both sets of data with the LIGAND program demonstrated a single class of high affinity receptor sites; the data would not fit a two-site model.

In contrast to their similar binding affinities, bPTH-(1-34) was substantially more potent than PTHLP-(1-36) in the canine renal cortical adenylate cyclase assay (Table 2). In the standard assay (30 min, 30 °C), bPTH-(1-34) had greater than 6-fold the potency of PTHLP-(1-36) with $K_0$ values of 0.06 and 0.40 nM, respectively. To exclude the possibility that selective destruction of PTHLP occurred during the assay in the presence of renal membranes, the adenylate cyclase assay was performed under binding conditions which had been demonstrated to result in negligible proteolysis of radioligands (see Table 1, Miniprint). Under conditions identical to the equilibrium binding assay (20 °C, 20 min, with bacitracin), adenylate cyclase stimulation by bPTH-(1-34) was 15-fold greater than for PTHLP-(1-36). The $K_0$ values under binding
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FIG. 5. Competition binding studies of $^{125}$I-(Tyr$^{61}$)PTHLP-(1–36)amide to canine renal membranes at 20 °C with unlabeled [Nle$^{618}$,Tyr$^{64}$]hPTH-(1–34)amide ( ), bPTH-(1–34) ( ), [Tyr$^{45}$]PTHLP-(1–36)amide ( ), PTHLP-(49–74) ( ), and [Cys$^{9}$,Trp$^{11}$]PTHLP-(5–18) ( . Points represent the mean ± S.E. of triplicate determinations in three separate experiments (bPTH-(1–34) and [Tyr$^{45}$] PTHLP-(1–36)amide) or in one experiment ([Nle$^{618}$,Tyr$^{64}$]hPTH-(1–34)amide). Individual points were expressed as a percentage of the specific binding determined in the absence of unlabeled peptide (percent of maximal specific binding). Scatchard analysis (inset) of a representative experiment is shown. B/F, bound/free.

FIG. 6. Photoaffinity covalent labeling of canine renal membranes isolated under limited proteolytic protection was visualized by autoradiography after SDS-polyacrylamide gel electrophoresis under reducing conditions. $^{125}$I-PTH refers to $^{125}$I-[Nle$^{618}$,Tyr$^{64}$]hPTH-(1–34)amide and $^{125}$I-PTHLP-(1–36) under reducing conditions (50 mM dithiothreitol) appeared as a broad band with a molecular mass of approximately 70 kDa. When using $^{125}$I-[Nle$^{618}$,Tyr$^{64}$]hPTH-(1–34)amide as the radioligand, binding at approximately 70, 25 (more evident at longer exposures of the autoradiogram), and 14 kDa could be abolished with an excess of unlabeled bPTH-(1–34) (Fig. 6, lanes 1 and 2). Covalent labeling of CRCM using $^{125}$I-PTHLP-(1–36) revealed a similar fluorographic pattern. Three bands similar to those displaced by unlabeled bPTH-(1–34) were displaced by the addition of excess unlabeled PTHLP-(1–36) (Fig. 6, lanes 3 and 4).

Photoaffinity cross-linking studies using CRCM isolated in the presence of multiple protease inhibitors (leupeptin, pepstatin, N-ethylmaleimide, aprotinin, and phenylmethylsulfonyl fluoride), as described by Nissenson et al. (17), revealed a major binding component at approximately 85 kDa using either radioligand. This cross-linking was fully inhibited by an excess of unlabeled bPTH-(1–34) or PTHLP-(1–36) (Fig. 7a). A minor binding species in excess of 130 kDa was apparent after longer exposures of the autoradiogram. Under reducing conditions (50 mM dithiothreitol), another minor component was evident at approximately 14 kDa (data not shown), which was not present under nonreducing conditions. Binding to both minor species was abolished by un-
centrations of bPTH-(1-34) or PTHLP-(1-36)amide (nanomolar).

labeled canine renal membranes isolated in the presence was seen at these PTH-like properties both in vivo in vitro. A two renal pathophysiologic features of HHM differ from primary hyperparathyroidism: 1) a presumed reduction in renal 1α-hydroxylase activity manifested by decreased plasma 1,25-dihydroxyvitamin D concentrations and 2) increased fractional calcium excretion (24, 25). While these observations may have several possible explanations, we have been interested in the possibility that there might be subclasses of PTH receptors in the kidney which manifest differing interactions with PTH and PTHLP. This possibility provided the impetus for the studies described herein.

The present studies have characterized the binding of the synthetic PTH-like peptide, PTHLP-(1-36)amide, and PTH analogues to canine renal membranes using high quality, monoidinated forms of the parent peptides as radioligand. The PTH analogue [Nle8,18,Tyr34]hPTH-(1-34)amide was chosen for radioiodination because it is resistant to oxidation during iodination. The human PTH sequence was selected for comparison to human PTHLP. The major peak of radioiodinated hormone was shown to be almost exclusively composed of the monoidinated form for both peptides, allowing accurate calculation of specific activity.

Significant metabolism of radioligand by canine renal membranes, even after purification on discontinuous sucrose gradients, occurred in the absence of protease inhibitors. This finding is somewhat at variance with that reported by Goltzman et al. (26), who found that purified CRCM did not degrade intact bPTH when incubated at 22 °C for 10 min. Segre et al. (16) similarly found no degradation of 125I-[Nle8,18,Tyr34]bPTH-(1-34)amide after incubation with CRCM for 1 h at 15 °C, while Rizzoli et al. (27) found only modest degradation of 125I-bPTH-(1-84) at 15 °C for 3 h using a similar membrane preparation. On the other hand, extensive degradation of radiolabeled PTH analogues by bovine (26), porcine (28), murine (29), chicken (30), and rabbit (31) renal membranes has been reported. Slight modifications in the technique for isolation of membranes and differences in incubation time and temperature may account for the greater degree of degradation which we observed. In the current study, the use of bacitracin during the binding studies prevented protease degradation of [Nle8,18,Tyr34]hPTH-(1-34)amide and PTHLP-(1-36), allowing analysis of binding properties free from artifacts introduced by degradation of the peptides.

Receptor binding using PTH analogues in the canine renal membrane system has been shown to be specific, saturable, and of high affinity, satisfying criteria for a receptor/hormone interaction (16, 30, 31). In the current study, comparison of two PTH analogues ([Nle8,18,Tyr34]hPTH-(1-34)amide and bPTH-(1-34)) with synthetic PTHLP-(1-36)amide demonstrated similar binding affinities for all three peptides when using either [Nle8,18,Tyr34]hPTH-(1-34)amide or PTHLP-(1-36) as radioligand. Competition curves of all three unlabeled peptides were nearly superimposable, although the derived binding affinity constant for PTHLP-(1-36) was slightly higher than for bPTH-(1-34). This difference (less than 2-fold) reached statistical significance only in studies using 125I-[Nle8,18,Tyr34]hPTH-(1-34)amide as the radioligand, but not in those using 125I-PTHLP-(1-36). Each unlabeled peptide

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reduced the binding of each radioligand to the same degree, suggesting that the peptides are binding to a similar or identical receptor. Scatchard analysis indicates a homogeneous class of high affinity receptor sites without significant cooperative binding interactions. Biologically inactive PTHLP fragments failed to displace the radioligand. These data, demonstrating similar binding affinities and $B_{\text{max}}$ values for PTHLP and PTH in renal membranes, are in agreement with a report which described similar binding affinities for the two peptides in bone-derived cells (32). A recent report also indicates similar binding affinities for PTH and PTHLP in canine renal membranes and UMR-106 osteosarcoma cells (33). Horii et al. (9) reported that PTHLP-(1-34) was approximately 10-fold less active than bPTH-(1-34) or hPTH-(1-34) in inhibiting binding of radiolabeled [Nle$^{8a}$,Tyr$^{34a}$]bPTH-(1-34)amide to bovine renal membranes. Insufficient data are provided in that study to carefully compare their methods to the current methods.

In contrast to its similar potency in the binding assay, PTHLP-(1-36) was significantly less potent (6-15-fold) in stimulating adenylyl cyclase in canine renal membranes when compared to bPTH-(1-34). This relationship was seen both in the standard assay (30°C for 30-min incubations) and under binding assay conditions (20°C for 20-min incubations) with the protease inhibitor bacitracin. These observations are in agreement with recent data by other investigators (9, 33). Thus, although the binding affinity of both peptides to the renal cortical receptor appears to be similar, bPTH-(1-34) appears to be more tightly coupled to post-receptor events required for adenylyl cyclase stimulation. The molecular basis for this relatively less efficient signaling is uncertain, but is of great interest. As in renal membranes, PTHLP and PTH appear to have identical binding characteristics to osteosarcoma cells (32, 33). In contrast to the cyclase response in canine renal membranes, PTHLP-(1-36) may be more potent in stimulating adenylyl cyclase than in inhibiting binding of their radiolabeled analogues. Other investigators have demonstrated using PTH analogues that the $K_m$ value for adenylyl cyclase stimulation was similar to the $K_d$ value in binding systems (16, 30, 31). These investigators have generally used adenylyl cyclase systems that lacked guanylate nucleotide amplification, resulting in $K_m$ values which were several fold higher than most sensitive renal adenylyl cyclase systems, such as those described herein (19, 34). Characterization of other receptor-cyclase coupling systems, such as the B-adrenergic receptor (35), indicate discrepant agonist concentrations for adenylyl cyclase stimulation and receptor binding, suggesting a “spare receptor” mechanism. The observed spare receptors may reflect the lowering of the $K_m$ by the addition of Gpp(NH)p to the adenylyl cyclase assay. Alternatively, the $K_d$ values observed might represent low affinity binding sites not linked to adenylyl cyclase.

Photoaffinity labeling of canine renal PTH receptors in membranes prepared in the presence of limited proteolytic protection (aprotinin and bacitracin) using the heterobifunctional cross-linking reagent hydroxysuccinimidyld-p-azidoenzoate indicates the presence of a predominant binding component of 70 kDa, while cross-linking of membranes prepared under more rigorous protease protective conditions, described by Niessenson et al. (17), yields an apparent 85-kDa receptor form. $^{125}$I-PTHLP-(1-36) and $^{125}$I-[Nle$^{8a}$,Tyr$^{34a}$]bPTH-(1-34)amide label indistinguishable components in the two membrane preparations. These results are analogous to those recently described by Nissenson and collaborators (33). Juppner et al. (32) have also demonstrated binding to an 80-kDa protein on intact ROS 17/28 osteosarcoma cells using photoactive derivatives of [Nle$^{8a}$,Tyr$^{34a}$]bPTH-(1-34)amide and PTHLP-(1-36), concluding that both peptides bind to the same receptor in these bone-derived cells. Two lower molecular mass binding components were also specifically labeled by both radioligands in membranes prepared under limited proteolytic protection. The lowest molecular mass form (less than or equal to 14 kDa) was also observed in the protected membranes, but disappeared under non-reducing conditions, suggesting that a portion of the intact receptor involves linkage with disulfide bonds.

Although amino-terminal PTHLP peptides appear to reproduce many of the features of HHM in vitro, the active physiologic form secreted by tumors is not yet known. Until the physiologically relevant secretory form(s) is characterized, the use of PTHLP-(1-36) or other synthetic PTHLP peptides as radioligands in competition binding studies should be interpreted with caution. These studies provide direct evidence that PTHLP-(1-36) and amino-terminal PTH analogues bind to identical or at least biochemically indistinguishable receptors in canine renal cortical membranes. These findings are consistent with the clinical and laboratory similarities between humoral hypercalcemia of malignancy and primary hyperparathyroidism at the renal level. The differences in bioactivity observed between the peptides is intriguing and appears to reflect differences in coupling to post-receptor events.

Acknowledgements—We thank Dr. John Perkins for his helpful criticism during these studies and Ann Blood for superb secretarial assistance.

REFERENCES


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Results

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Radioligand Precipitation and Analysis: Separation of labeled peptides after radioligand precipitation is shown in Figure 1. Radiolabeled IgG1,TyryzI, TyryzI (Gly8)-Protein-PTH(1-34) (a) was purified from the water phase of radioligand precipitation by high-performance liquid chromatography (HPLC). The radioactivity was recovered from the radioligand from Figure 1(a). Similarly, radioactivity from the water phase of radioligand precipitation was collected to determine the specific activity of the radioligand. The specific activity of the radioligand was calculated from the mean specific activity of the internal standard, assuming complete separation from the peptide (as demonstrated by HPLC chromatography and the absence of any visible impurities in the peptide-based assay). The results showed that the specific activity of the radioligand was 0.08 mg/mg (Figure 1(a)).

Specificity of Binding: Inhibition of the radioligand by unlabeled peptide was determined by a Scatchard analysis. The results showed that the IC50 of the unlabeled peptide was 0.005 mg/mg (Figure 1(b)). Inhibition of binding was also observed in the presence of unlabeled peptide at concentrations of 10-50 mg/mg (Figure 1(c)). Inhibition of binding was also observed in the presence of unlabeled peptide at concentrations of 10-50 mg/mg (Figure 1(c)).

Characterization of Ligand Binding: The specificity of the radioligand binding was determined by Scatchard analysis. The specific activity of the radioligand was calculated from the mean specific activity of the internal standard, assuming complete separation from the peptide (as demonstrated by HPLC chromatography and the absence of any visible impurities in the peptide-based assay). The results showed that the specific activity of the radioligand was 0.08 mg/mg (Figure 1(a)).

Figure 1. a. Radiolabeled IgG1,TyryzI, TyryzI (Gly8)-Protein-PTH(1-34) (a) was purified from the water phase of radioligand precipitation by high-performance liquid chromatography (HPLC). b. Inhibition of binding by unlabeled peptide was determined by a Scatchard analysis. The specific activity of the radioligand was calculated from the mean specific activity of the internal standard, assuming complete separation from the peptide (as demonstrated by HPLC chromatography and the absence of any visible impurities in the peptide-based assay). The results showed that the specific activity of the radioligand was 0.08 mg/mg (Figure 1(a)).

Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding (IC50)</th>
</tr>
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<tbody>
<tr>
<td>IgG1,TyryzI, TyryzI (Gly8)-Protein-PTH(1-34)</td>
<td>0.005 mg/mg</td>
</tr>
<tr>
<td>IgG1,TyryzI, TyryzI (Gly8)-Protein-PTH(1-34)</td>
<td>0.08 mg/mg</td>
</tr>
</tbody>
</table>

Table I. Analysis of radioligand after exposure to canine renal membranes for 10 min at 37°C under binding assay conditions in the presence of the protein substrate, horseradish peroxidase (HRP). The specific activity of the radioligand was calculated from the mean specific activity of the internal standard, assuming complete separation from the peptide (as demonstrated by HPLC chromatography and the absence of any visible impurities in the peptide-based assay). The results showed that the specific activity of the radioligand was 0.08 mg/mg (Figure 1(a)).