Covalent Labeling of a High-Affinity, Guanyl Nucleotide Sensitive Parathyroid Hormone Receptor in Canine Renal Cortex†

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ABSTRACT: Putative parathyroid hormone (PTH) receptors in canine renal membranes were affinity labeled with ^{125}I-bPTH(1–34) using the heterobifunctional cross-linking reagent *N*-hydroxysuccinimidyl 4-azidobenzoate. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed the presence of a major 85 000 molecular weight (M_r) PTH binding component, the labeling of which was inhibited by nanomolar concentrations of unlabeled PTH and by micromolar concentrations of 5′-guanylyl imidodiphosphate [Gpp(NH)p]. Labeling was not influenced by the unrelated peptides insulin and arginine vasopressin. Minor PTH binding components of M_r 55 000 and 130 000 were also seen, and labeling of these was likewise sensitive to unlabeled PTH and to Gpp(NH)p. Omission of protease inhibitors during the isolation of plasma membranes resulted in the loss of the M_r 85 000 PTH binding species and the appearance of an M_r 70 000 form. Several minor PTH binding components also were observed. Equilibrium binding studies showed that such membranes had an affinity for PTH indistinguishable from that in membranes isolated with protease inhibitors and displaying a major M_r 85 000 PTH binding species. We conclude that the major form of the adenylate cyclase coupled PTH receptor in canine renal membranes is an M_r 85 000 protein. An endogenous enzyme, probably a lysosomal cathepsin, can cleave this form to produce an M_r 70 000 receptor that retains full functional activity with respect to high-affinity, guanyl nucleotide sensitive PTH binding. The ability to covalently label the PTH receptor in high yield represents a major step toward the structural characterization of this important detector molecule.

Parathyroid hormone (PTH)[1] receptors on target cells in bone and kidney initiate the biological effects of at least two humoral factors. PTH itself binds to receptors in bone (Pliam et al., 1982; Silve et al., 1982; Rizzoli et al., 1983a; Rao & Murray, 1985) and kidney (Zull et al., 1977; Nissenson & Arnaud, 1979; Segre et al., 1979; Bellorin-Font & Martin, 1981; Teitelbaum et al., 1982; Kremer et al., 1982; Forte et al., 1982; Rizzoli et al., 1983b; Teitelbaum & Strewler, 1984; McKee & Murray, 1985), thereby activating the adenylate cyclase–cyclic AMP dependent protein kinase cascade, leading to cellular responses. In addition, investigators recently have identified a tumor-derived factor immunochemically distinct from PTH but capable of activating renal and skeletal PTH receptors (Strewler et al., 1983; Stewart et al., 1983; Rodan et al., 1983; Nissenson et al., 1985a). This factor has been proposed as a possible humoral mediator of hypercalcemia associated with certain solid malignancies.

Despite widespread interest in the mechanisms of action of PTH, little data are available on the structural properties of PTH receptors. Coltrera et al. (1981) and Draper et al. (1982) have developed iodinated photoreactive analogues of [Nle,-Nle,^{18}Tyr^{34}]bPTH(1–34)amide and have identified PTH binding components with a molecular weight of 60 000–70 000 in canine renal plasma membranes. Goldring et al. (1984) identified a PTH binding protein of similar size in PTH-responsive cultured human skin fibroblasts and giant cell tumors of bone. Although these binding components had certain properties expected of physiological receptors, the concentration of unlabeled PTH used to inhibit photoaffinity labeling ($\sim$1.0 μM) was extremely high when compared to the published affinity of the adenylate cyclase coupled canine renal PTH receptor [$K_d \leq 5$ nM (Teitelbaum et al., 1982; Martin et al., 1985)]. The possibility that these PTH binding sites identified by photoaffinity labeling may represent low-affinity components raises a question as to their physiological relevance.

In the present study, we have used the heterobifunctional cross-linking reagent HSAB to covalently label canine renal PTH receptors. The results indicate the presence in native membranes of a predominant PTH binding component of M_r 85 000, the labeling of which is inhibited completely by low concentrations of unlabeled PTH (24 nM). This high-affinity PTH binding component is converted to a high-affinity M_r 70 000 form by the action of an endogenous renal proteolytic enzyme.

Materials and Methods

Materials. Phenylmethanesulfonyl fluoride, pepstatin, leupeptin, and NEM were purchased from Sigma Chemical Co. (St. Louis, MO). Gpp(NH)p, GMP, GDPβS, and App(NH)p were from Boehringer Mannheim Biochemicals (Indianapolis, IN), HSAB was from Pierce Chemical Corp. (Rockford, IL), and aprotinin was from Mobay Chemical Corp. (New York, NY). Bovine PTH(1–34) (5000 units/mg)

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[1] Abbreviations: PTH, parathyroid hormone; M_r, molecular weight; HSAB, *N*-hydroxysuccinimidyl 4-azidobenzoate; b, bovine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid trisodium salt; GTP, guanosine 5′-triphosphate; GMP, guanosine 5′-monophosphate; Gpp(NH)p, 5′-guanylyl imidodiphosphate; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); App(NH)p, 5′-adenylyl imidodiphosphate; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride.

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and arginine vasopressin were from Bachem (Torrance, CA).

Isolation of Canine Renal Plasma Membranes. Canine renal cortical plasma membranes were isolated by a published modification (Nissenson & Arnaud, 1979) of the technique of Fitzpatrick et al. (1969). Such preparations are enriched 7–10-fold (over crude homogenates) in ouabain-sensitive (Na^+,K^+)-ATPase. In experiments designed to evaluate the effects of endogenous proteases, a single kidney was processed in buffers containing the relevant protease inhibitors, whereas the contralateral kidney was processed without protease inhibitors and served as a "control". Membranes were stored at −80 °C and were stable with respect to ^{125}I-bPTH(1–34) binding activity for at least 6 months.

^{125}I-bPTH(1–34) Receptor Cross-Linking. Biologically active, electrolytically labeled ^{125}I-bPTH(1–34) was prepared and purified by high-performance liquid chromatography as previously described (Nissenson et al., 1986). Approximately 1.0 μCi of ^{125}I-bPTH(1–34) was incubated for 2 h at 30 °C with canine renal membranes (250 μg) in 1.0 mL of a solution consisting of 50 mM Tris-HCl, 50 mM HEPES (pH 7.5), 2.0 mM $MgCl_2$, and 0.1% BSA. Unlabeled bPTH(1–34), other peptides, or Gpp(NH)p was added as indicated. Incubates were microcentrifuged at 4 °C, and the pellets were resuspended in ice-cold 50 mM sodium phosphate (pH 7.6) containing 0.1% BSA. After centrifugation, this wash step was repeated. Membrane pellets were washed an additional time with 50 mM sodium phosphate (pH 7.6, no BSA) and were resuspended in 350 μL of this buffer. Seven microliters of the heterobifunctional cross-linking reagent HSAB (6.5 mg/mL in dimethyl sulfoxide) was added, and incubation was carried out for 10 min on ice in the dark. The reaction was terminated by the addition of 7 μL of 2 M Tris-HCl (pH 7.5). Samples were then transferred to a single well of a 24-well cluster plate and subjected to photolysis for 20 min using a Blak-Ray ultraviolet lamp (emission maximum 365 nm) at a distance of 8 cm. Samples were than transferred to microfuge tubes, and the membranes were washed twice with 50 mM sodium phosphate (pH 7.6). The efficiency of covalent bond formation was 5–10% of the bound ^{125}I-bPTH(1–34).

SDS–PAGE. Membrane pellets were run on SDS–PAGE by the method of Laemmli (1970) using 5–15% linear gradient gels. Membrane samples were heated at 37 °C for 10 min in sample buffer containing 1.0% SDS and 30 mM dithiothreitol before electrophoresis. Boiling samples at this point induced excessive aggregation of labeled membrane components as indicated by their failure to enter the resolving gel. Hence, boiling was avoided. Molecular weight markers were run on parallel lanes. Gels were stained with Coomassie blue, destained, dried, and developed for autoradiography using X-Omat R film (Kodak, Rochester, NY) with Chronex Lightning-Plus intensifying screens (Dupont, Wilmington, DE).

Receptor Binding Assays. PTH receptors in canine renal membranes were quantified in competitive displacement experiments using ^{125}I-bPTH(1–34) as described (Nissenson et al., 1985b), except that bound hormone and free hormone were separated by microcentrifugation rather than by filtration. Calculations of PTH receptor number and affinity were by Scatchard analysis (Scatchard, 1949) of competitive binding experiments in which 10 competing doses of bPTH(1–34) were used, and ^{125}I-bPTH(1–34) binding at each dose was assessed in triplicate. With the use of a nonlinear regression model (SAS Institute Inc., Cary NC), these data were best fit to a single homogeneous binding site lacking cooperativity, as previously described (Teitelbaum et al., 1982). The standard error of the estimates of the number and affinity of PTH receptors was determined by analysis of variance.

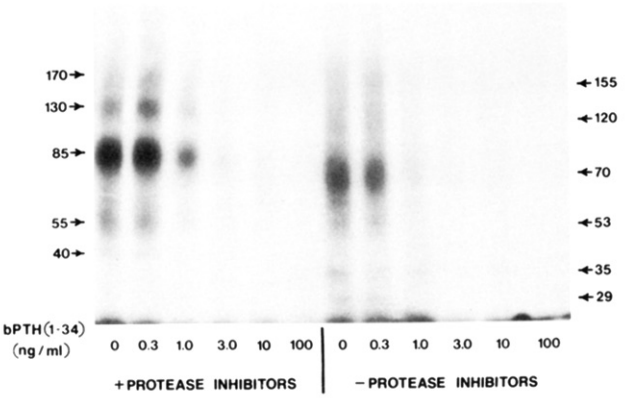

FIGURE 1: Autoradiogram of SDS–PAGE of canine renal plasma membranes after cross-linking ^{125}I-bPTH(1–34) to putative receptor components. Renal membranes were isolated either in the presence or in the absence of the following combination of protease inhibitors: PMSF (10 μg/mL), leupeptin (5.0 μg/mL), pepstatin (5 μg/mL), aprotinin (10 units/mL), and *N*-ethylmaleimide (1.0 mM). The concentrations of unlabeled bPTH(1–34) present during the binding reaction are presented below the autoradiogram. The calibrated molecular weights of the labeled components are indicated with arrows.

RESULTS

Canine renal plasma membranes, isolated in the absence of protease inhibitors other than EDTA (1.0 mM), displayed multiple PTH binding components (Figure 1). Consistently, the major ^{125}I-bPTH(1–34)–protein complex appeared as a broad band of apparent M_r ~70 000. Additional labeled components were seen with apparent molecular weights of ~155 000, 120 000, 70 000, 53 000, 35 000, and 29 000. Plasma membranes were isolated from the contralateral kidney in the presence of multiple protease inhibitors. The predominant PTH binding component in these membranes had an apparent molecular weight of ~85 000. Less intense labeling was also evident at M_r 130 000 and 55 000. Faint bands were apparent at M_r 170 00 and 40 000. Labeling of these components persisted when photolysis was carried out in the presence of 1.0 and 10 mM *p*-aminobenzoate, a nitrene scavenger (data not shown).

The apparent affinity of these components for PTH was assessed by including various concentrations of unlabeled bPTH(1–34) in the binding reaction before affinity cross-linking (Figure 1). Labeling of all components was partially inhibited by 1.0 ng/mL (0.24 nM) and abolished by 100 ng/mL (24 nM) bPTH(1–34). Conventional radioligand binding assays revealed inhibition of specific ^{125}I-bPTH(1–34) binding to renal membranes over the same concentrations of unlabeled bPTH(1–34) (Figure 2).

The selectivity of the ^{125}I-bPTH(1–34) binding sites was assessed in membranes isolated in the presence of protease inhibitors. Native bPTH(1–84) (1.1 μM) and human PTH-(1–34) (0.24 μM) abolished both ^{125}I-bPTH(1–34) binding to membranes and covalent binding of all components on SDS–PAGE. Insulin (0.17 μM) and arginine vasopressin (1.0 μM) did not affect membrane binding and covalent labeling (data not shown).

We previously have reported that the binding affinity of adenylate cyclase coupled PTH receptors in canine renal membranes is reduced when GTP (or its analogues) is present during the binding reaction (Teitelbaum et al., 1982). In the present study, Gpp(NH)p (10 nM–1.0 μM) markedly inhibited specific ^{125}I-bPTH(1–34) binding to renal membranes isolated both with and without protease inhibitors (Figure 3).

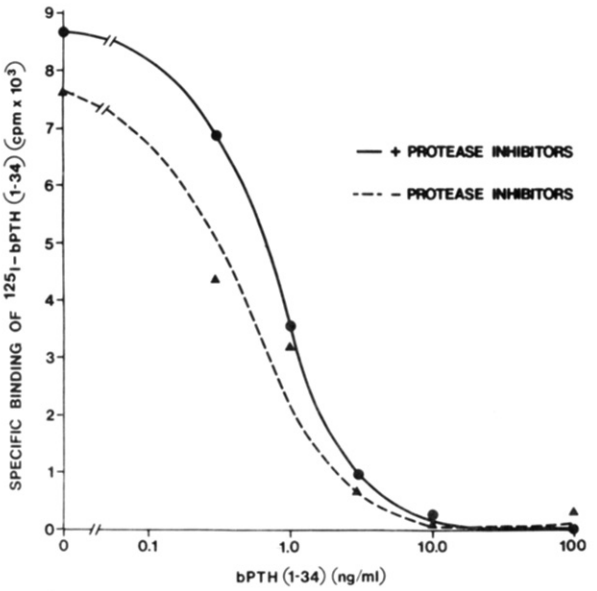

FIGURE 2: Competitive binding of bPTH(1–34) to receptors in canine renal plasma membranes in the absence (---) or presence (—) of the protease inhibitors listed in the legend to Figure 1. In the experiment described in Figure 1, an aliquot of membranes was removed from each sample for assessment of bound ^{125}I-bPTH(1–34) before cross-linking. The IC_{50} values for bPTH(1–34) were 1.0 ng/mL (0.24 nM) and 0.8 ng/mL (0.19 nM) for membranes isolated respectively in the presence and absence of protease inhibitors.

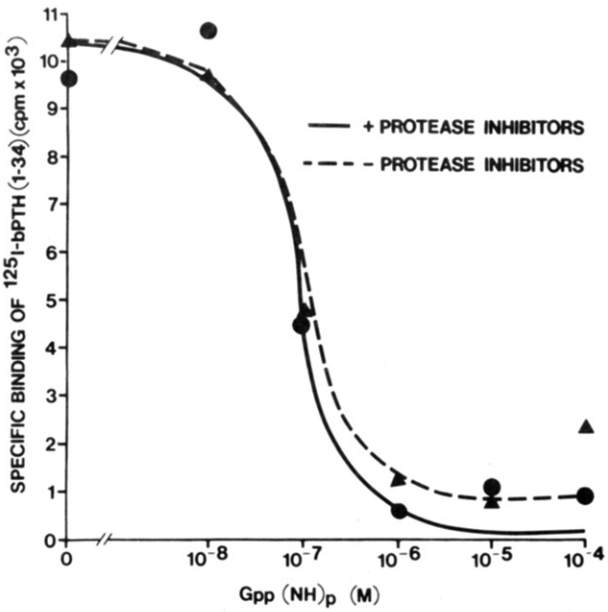

FIGURE 3: Effect of Gpp(NH)p on steady-state ^{125}I-bPTH(1–34) binding to canine renal membranes isolated in the presence and absence of protease inhibitors (see legend to Figure 1). The IC_{50} values for Gpp(NH)p were 100 nM in both sets of membranes.

At the same concentrations, Gpp(NH)p inhibited covalent labeling of all membrane components on SDS–PAGE (Figure 4). Inhibition of labeling was also produced with the GDP analogue GDPβS (100 μM) but not with the inactive nucleotides App(NH)p (100 μM) or GMP (100 μM) (data not shown).

Further studies were undertaken to characterize the proteolytic activity presumed to be responsible for converting the major form of the PTH receptor from a protein of M_r 85 000 to one of M_r 70 000. The M_r 85 000 PTH receptor component was perserved only when protease inhibitors were present during homogenization and renal membrane isolation. Membranes that were isolated in the absence of protease inhibitors, but stored and labeled with ^{125}I-bPTH(1–34) in the presence of inhibitors, displayed a major M_r 70 000 binding component (data not shown). It is thus likely that the enzyme responsible for degrading the PTH receptor is of endogenous renal origin. When individual protease inhibitors were tested, NEM (1.0 mM) and leupeptin (45 μg/mL) partially reproduced the effect of the combined inhibitors (Figure 5).

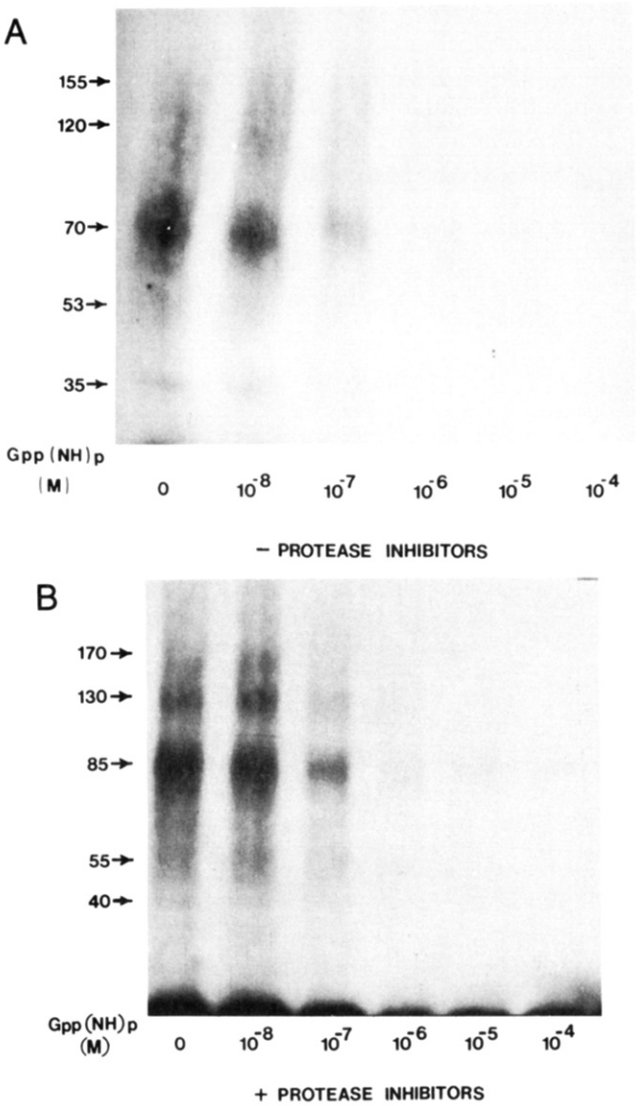

FIGURE 4: Effect of Gpp(NH)p on covalent ^{125}I-bPTH(1–34) binding to proteins in canine renal membranes isolated in the absence (A) or presence (B) of protease inhibitors (see legend to Figure 1).

The results shown in Figure 2 suggest that conversion of PTH receptors from an M_r 85 000 to an M_r 70 000 form did not inactivate the receptor or reduce its affinity for PTH. Detailed competitive binding experiments in membranes made with inhibitors and displaying the M_r 85 000 form vs. control membranes from contralateral kidneys displaying the M_r 70 000 form confirmed this result (Table I). In all cases, data were best fit to a single-site, noncooperative receptor model. Membranes expressing predominantly the M_r 85 000 species showed an affinity and a PTH binding capacity virtually identical with those expressing the M_r 70 000 form.

Discussion

Despite general agreement that the adenylate cyclase coupled PTH receptor initiates the hormone's effects on the proximal renal tubule, only limited data are available concerning the structure of this important receptor. Coltrera et al. (1980) synthesized two photoaffinity probes consisting of

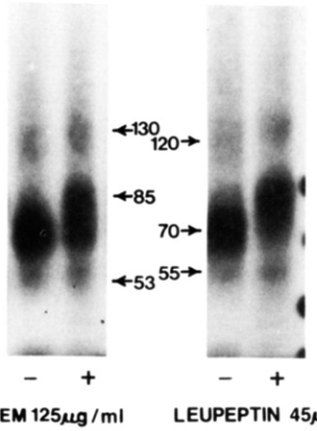

FIGURE 5: Covalent cross-linking of ^{125}I-bPTH(1–34) to receptors in canine renal membranes isolated in the absence of protease inhibitors or in the presence of either high-dose leupeptin (45 μg/mL) or NEM (125 μg/mL = 1.0 mM).

Table I: Number and Affinity of PTH Receptors in Canine Renal Plasma Membranes Isolated in the Presence and Absence of Protease Inhibitors

expt	protease inhibitor	major PTH receptor species M_r	PTH receptor affinity, K_d (ng/mL)	PTH receptor no. (pmol/mg)
1	none	70000	1.7 ± 0.3	0.25 ● 0.03
	all inhibitors[a]	85000	1.8 ± 0.6	0.22 ● 0.03
2	none	70000	1.4 ± 0.2	0.17 ± 0.02
	leupeptin (45 μg/mL)	85000	1.1 ● 0.1	0.15 ± 0.02
3	none	70000	1.5 ● 0.2	0.21 ± 0.02
	leupeptin (45 μg/mL)	85000	1.3 ± 0.2	0.15 ● 0.02
4	none	70000	1.5 ± 0.2	0.21 ± 0.02
	NEM (1.0 mM)	85000	1.3 ● 0.2	0.15 ± 0.02

[a] PMSF (10 μg/mL), leupeptin (5.0 μg/mL), pepstatin (5 μg/mL), aprotinin (10 units/mL), and NEM (1.0 mM).

^{125}I-[Nle,^{8}Nle,^{18}Tyr^{34}]bPTH(1–34) amide derivatized with the amino group specific photolabile reagents 4-fluoro-3-nitrophenyl azide and *N*-succinimidyl 6-[(4-azido-2-nitrophenyl)-amino]hexanoate. Both photoaffinity probes covalently labeled multiple protein components of canine renal plasma membranes. Labeling of one of these components (M_r ~70000) was inhibited in the presence of excess unlabeled. [Nle,^{8}Nle,^{18}Tyr^{34}]bPTH(1–34) amide (24 μM). Draper et al. (1981) derivatized the same radioligand at position 23 tryptophan with the photoreactive reagent (2-nitro-5-azidophenyl)sulfenyl chloride and identified two PTH binding proteins in canine renal membranes. The labeling of one of these (M_r ~60000) was inhibited by unlabeled bPTH(1–34) (2.4 μM). In neither study was the PTH binding component shown to possess the high affinity for PTH and the sensitivity to GTP analogues that would be expected based upon the known properties of adenylate cyclase coupled PTH receptors in canine renal membranes.

In the present study, photoaffinity cross-linking with HSAB enabled identification of a major PTH binding protein of M_r ~85000 in canine renal membranes isolated in the presence of protease inhibitors.[2] Several lines of evidence support the conclusion that this protein is a constituent of the adenylate cyclase coupled PTH receptor. First, the M_r 85000 protein displayed an apparent affinity for bPTH(1–34) indistinguishable from that previously reported for the adenylate cyclase coupled PTH receptor in canine renal membranes (Teitelbaum et al., 1982). Second, labeling of the M_r 85000 protein was inhibited only by peptides known to interact with the PTH receptor but not by insulin or arginine vasopressin. Third, Gpp(NH)p inhibited labeling of the M_r 85000 protein over the same concentrations required to bind to the stimulatory GTP binding component of adenylate cyclase, Gs (Northrup et al., 1982), and to decrease high-affinity PTH binding in solubilized canine renal cortical membranes (Nissenson et al., 1986).

Although the major PTH binding component appears to be an M_r 85000 protein, we cannot rule out the possibility that this species represents a lower molecular weight receptor covalently coupled to a neighboring membrane protein. If so, the covalent association of receptor with this putative protein must be very efficient. It will be important to confirm the size of the binding moiety of the PTH receptor using independent techniques (e.g., antireceptor antibodies).

In addition to the major M_r 85000 component, covalent binding of ^{125}I-bPTH(1–84) to minor components of M_r 55000 and 130000 was consistently observed despite the presence of protease inhibitors during membrane isolation. This heterogeneity did not diminish when protease inhibitors were also present during both the storage of membranes at −80 °C and the incubation of membranes with ^{125}I-bPTH(1–34). Both of these proteins bound bPTH(1–34) with high affinity, and both were sensitive to Gpp(NH)p. These minor components are thus specific binding proteins that are presumably constituents of, or closely related to, the PTH receptor. It is likely that the M_r 55000 species is a proteolytic product of the major M_r 85000 form. In support of this, we have recently observed a time-dependent loss of the M_r 85000 form with increased labeling of the M_r 55000 form in membranes solubilized with Triton X-100 (R. A. Nissenson, unpublished data). The relationship of the M_r 130000 component to these other forms is uncertain. It may represent the M_r 85000 component covalently associated with a neighboring membrane protein (e.g., the M_r 55000 receptor species). Alternatively, the M_r 130000 component may be a protein, proximate to the receptor, to which ^{125}I-bPTH(1–34) became covalently associated during photolysis. We favor the former interpretation inasmuch as the M_r 85000 and 130000 forms show a parallel 10000–15000 molecular weight decrease in membranes isolated in the absence of protease inhibitors. We cannot rule out the additional possibility that the M_r 85000 and 55000 forms are both proteolytic fragments of an intact M_r 130000 receptor. Detailed peptide mapping studies will be required to establish these relationships with certainty.

The presence of a major M_r 70000 PTH receptor species in membranes prepared without protease inhibitors is most readily explained by the presence of an endogenous enzyme capable of converting the M_r 85000 form to an M_r 70000 form. Although the relationship of this M_r 70000 component to the M_r 60000–70000 PTH binding species previously identified by photoaffinity labeling is uncertain, it is noteworthy that in the studies of Coltrera et al. (1981) and Draper et al. (1982) protease inhibitors were not used during the preparation of canine renal cortical plasma membranes. In the present study, the putative PTH receptor protease was inhibited by a combination of protease inhibitors (phenylmethanesulfonyl fluoride, leupeptin, NEM, pepstatin, and aprotinin). However, only NEM (1 mM) and a high concentration of leupeptin (45 μg/mL) were effective individually. At a lower concentration

[2] Molecular weight estimates for receptor components correspond to the actual position of labeled bands on SDS–PAGE without correction for the contribution of ^{125}I-bPTH(1–34) and HSAB. Assuming a 1:1:1 stoichiometry between ^{125}I-bPTH(1–34)/HSAB/binding protein, the estimates of receptor molecular weights would be ~4500 less than those presented.

(5 μg/mL), leupeptin by itself was ineffective (data not shown). These results lead us to conclude tentatively that the responsible enzyme is a lysosomal cysteine protease. In this class of proteases, the relatively low sensitivity to leupeptin is a property displayed only by cathepsin H (Barrett & Kirschke, 1981).

In summary, an M_r 85000 protein is the major form of the adenylate cyclase coupled PTH receptor in canine renal cortical membranes. An endogenous enzyme with properties similar to cathepsin H can cleave this form to produce an M_r 70000 receptor that retains full activity with respect to high-affinity, guanyl nucleotide sensitive PTH binding. The ability to reproducibly and selectively label the PTH receptor covalently in high yield should enable rapid structural characterization of this important detector molecule.

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Registry No. PTH, 9002-64-6; bPTH(1–34), 12583-68-5; Gpp(NH)p, 34273-04-6.

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