Metabolism and Biological Activity of Parathyroid Hormone in Renal Cortical Membranes

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ABSTRACT Recent studies from several laboratories have documented the presence of fragments of parathyroid hormone in blood or peripheral tissues or in both. Inasmuch as amino-terminal fragments are known to be biologically active, it has been suggested that fragments, rather than the intact polypeptide of 84 amino acids, might be the active molecular species in tissue fluids.

Accordingly, the metabolism of native bovine parathyroid hormone, bPTH-(1–84), was studied in purified renal cortical membranes from several species and correlated with hormonal stimulation of adenylyl cyclase in these membranes in vitro. Analysis of whole incubation mixtures or membrane-bound hormone by gel electrophoresis and gel chromatography after incubation of [^{3}H]bPTH-(1–84) or ^{125}I-labeled bPTH-(1–84) or unlabeled biologically active bPTH-(1–84) with purified canine renal cortical membranes revealed no evidence of proteolysis, and yet the uncleaved hormone readily stimulated adenylyl cyclase. Kinetic studies of hormone-stimulated adenylyl cyclase activity revealed no difference in rate of onset of activity between bPTH-(1–84) and the active synthetic amino-terminal tetratriacontapeptide bPTH-(1–34), and hence there was no evidence of precursor-product relationship between the native hormone and an active amino-terminal fragment.

The results suggest, insofar as the activity detected in these membranes reflects the biological response of the hormone in vivo, that the native hormone is indeed biologically active at the receptor level directly without the requirement for cleavage into active fragments.

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INTRODUCTION

Parathyroid hormone (PTH)[1] is an 84-amino-acid, single-chain polypeptide (1–3) with virtually full biological activity residing within the amino-terminal 34 residues of the molecule (4–6). Immunoassay studies of the circulating forms of PTH in man (7–14) and cow (8) have demonstrated heterogeneity resulting from the presence of hormonal fragments in the circulation. The predominant circulating entity seems to be biologically inactive, consisting of the middle and carboxyl-terminal portions (10, 12, 13, 15) of the hormone. It was reported that an amino-terminal fragment with the necessary requirements for biological activity exists in the circulation of man (16); a similar or identical fragment is apparently capable of being generated in peripheral tissues (15, 17–19). These findings have led to the suggestion by several groups (15, 18, 20) that the amino-terminal fragment(s), rather than the glandular intact 84-amino-acid form of the hormone, might be the form(s) of the molecule responsible for the hormone-specific effects on target tissues. The purpose of the studies described here was to determine in systems in vitro whether biological activity (stimulation of adenylyl cyclase) could be observed in the absence of cleavage of the native hormone to an independent active fragment.

METHODS

Hormones. The 1–84 bPTH I [about 3,000 Medical Research Council (MRC) U/mg] was extracted from bovine parathyroid glands and purified as previously described (1). Native bPTH-(1–84), used as a standard in the adenylyl cyclase assay for potency determination, was MRC Research Standard, Lot MRC 72/286 (National Institute for Medical

[1] *Abbreviations used in this paper:* cAMP, cyclic 3',5'-adenosine monophosphate; bPTH, bovine parathyroid hormone; GP, guinea pig; hPTH, human parathyroid hormone; MRC, Medical Research Council, Great Britain; PTH, parathyroid hormone.

Research, Mill Hill, London, England). bPTH-(1–34) was synthesized as previously described (4).

[^{3}H]bPTH-(1–84), [^{14}C]bPTH-(1–84), and [^{35}S]bPTH-(1–84) were isolated from bovine parathyroid gland slices incubated with [^{3}H]leucine, ^{14}C-labeled amino-acid mixtures, and [^{35}S]methionine, respectively. Details of the incubation, extraction, and separation methods have been described (21). To prevent oxidation of the hormones, polyacrylamide gels used in the purification procedure were pre-electrophoresed and aged in water for 48 h to permit outward diffusion of ammonium persulfate. bPTH-(1–84) and bPTH-(1–34) were iodinated with either ^{125}I or ^{131}I by the method of Hunter and Greenwood (22), employing Quso G32 (Philadelphia Quartz Co., Philadelphia, Penn.) for purification followed by gel filtration on Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, Calif.). The specific activity of ^{125}I-bPTH-(1–84) was 300 μCi/μg.

Membranes. Kidneys were obtained from male Sprague-Dawley rats, 150–200 g, from calves 7–10 days old, and from mongrel dogs, 20–27 kg, and highly purified renal cortical membranes were prepared by the method of Marx et al. (23). The renal cortex was dissected free and homogenized at 4°C in a 30% (vol/vol) buffer solution of 0.25 M sucrose, 0.01 M Tris, pH 7.5, and 0.001 M Na_2EDTA. The homogenized tissue was then diluted 1:1 with the buffer solution and spun in a Sorvall RC-2B ultracentrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.) until the rotor (SS-34) speed reached 4,500 rpm (2,200 *g*). The supernatant fluid was collected, and the centrifugation was repeated. The supernate of this second spin was then centrifuged at 4,500 rpm for 15 min, and the upper portion of the resulting double-layered pellet was resuspended in the previously described buffer solution, rehomogenized, and recentrifuged at 4,500 rpm for 15 min. The resulting pellet was resuspended in a small volume of the buffer solution and layered onto continuous 11-ml linear gradients of 32%–42% (wt/wt) sucrose in 0.01 M Tris, pH 7.5, 0.001 M Na_2EDTA. Gradients were centrifuged for 60 min at 25,000 rpm in the SW41 rotor of a Beckman L2-65B ultracentrifuge at 4°C (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). After centrifugation, two major bands of turbidity in addition to a pellet were readily identified with preparations from each species. The upper (38% sucrose) band was aspirated with a Pasteur pipette, diluted with 3 vol of a buffer solution of 0.01 M Tris, pH 7.5, 0.001 M Na_2EDTA, and centrifuged for 15 min at 4,500 rpm. The pellet (purified membrane fraction) was then resuspended in 0.05 M Tris, pH-7.5, and centrifuged at 2,200 *g* for 15 min in small aliquots. Pellets (containing 350–950 μg protein) were either used immediately or stored at −70°C without detectable loss of adenylyl cyclase activity for at least 8 wk. These purified membranes are primarily plasma or antelumenal membranes (23), enriched in Na/K-ATPase and adenylyl cyclase activity, and decreased in specific activity of marker enzymes for endoplasmic reticulum and brush border (23).

Incubation of hormone with membranes and analysis by gel electrophoresis. Biosynthetic bPTH-(1–84), internally labeled with [^{3}H]leucine, was incubated for 10 min at 22° or 37°C with approximately 50 μg of membrane protein and 0.25% bovine serum albumin in 0.05 M Tris-HCl, pH 7.4, in a total incubation volume of 100 μl. The incubation was stopped by precipitating the proteins with 15% trichloroacetic acid and centrifuging; the protein pellet was resuspended in 1 ml of H_2O and lyophilized. In preliminary experiments, it was determined that more than 95% of a fragment of bPTH as small as ^{125}I-labeled bPTH-(1–34) could be precipitated with as little as 10% trichloroacetic acid. The protein was then extracted with 8 M urea–0.1 M acetic acid and analyzed as previously described (21) by electrophoresis on urea-polyacrylamide gels at pH 4.4 that contained 10% acrylamide-0.25% bisacrylamide. Biosynthetic internally labeled [^{14}C]bPTH was used as a marker. Gels were cut into 1-mm slices and incubated overnight with 1 ml of a solution containing NCS Tissue Solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.) water: scintillation fluid (0.4:0.04:0.56) and assayed for radioactivity.

Unlabeled native bPTH-(1–84), 0.5 μM, was incubated for 10 min at 22°C in a total volume of 100 μl containing 50 μg of membrane protein and the solution described under *Assay of adenylyl cyclase activity*. At the end of the incubation, the sample was thoroughly mixed. 80 μl was assayed for adenylyl cyclase activity, and 20 μl was analyzed either by gel chromatography (to be described later) or by electrophoresis on polyacrylamide gels with ^{125}I-labeled bPTH-(1–84) and ^{125}I-labeled bPTH-(1–34) as internal markers. Gels were then cut into 1-mm slices, and each slice was shaken for 24 h at 4°C in 200 μl of a solution of 0.05 M barbital buffer (pH 8.5) with 10% (vol/vol) human plasma. The samples were then assayed for ^{125}I-radioactivity and analyzed for PTH by radioimmunoassay with ^{125}I-labeled bPTH with bPTH-(1–84) as a standard. The antigenic determinants recognized by guinea pig 1 (GP-1) antiserum used in these studies were previously characterized and are known to include portions of both the NH_2- and COOH-terminal regions of bPTH-(1–84) (10).

Incubation of hormone with membranes and analysis by gel chromatography. [^{3}H]bPTH-(1–84) (4,500 cpm, 12 mU) or ^{125}I-labeled bPTH-(1–84) (130,000 cpm, 390 pg) was incubated for 10 min at 22°C with approximately 50 μg of membrane protein in 0.05 M Tris-HCl buffer, pH 7.4, in a total incubation volume of 100 μl. Hormone was incubated with active or heat-inactivated (10 min, 65°C) membranes. 500 μl of 8 M guanidine hydrochloride (Heico Inc., Delaware Water Gap, Penn.) was added to stop the reaction and solubilize the membranes. [^{35}S]bPTH-(1–84) was added in 50 μl of 8 M guanidine to the incubation mixtures containing ^{3}H radioactivity to mark the elution position of intact hormone, and ^{131}I-labeled bPTH-(1–84), ^{131}I-labeled bPTH-(1–34), and Na^{131}I were added in 50 μl 20% acetone–1% acetic acid to the incubation mixtures containing ^{125}I radioactivity to mark the elution positions of intact hormone, active fragment, and the salt peak. The columns used for ^{3}H and ^{35}S radioactivity were subsequently calibrated with ^{125}I-labeled bPTH-(1–34) and Na^{125}I. The solubilized incubation contents originally containing biosynthetic bPTH or iodine-labeled bPTH were then chromatographed by gel filtration on 1.2 × 85-cm or 1.2 × 70-cm columns, respectively, of Bio-Gel A-0.5m (100–200 mesh) (Bio-Rad Laboratories) at 26°C with an eluting buffer of 6 M guanidine hydrochloride-0.1 M ammonium acetate-1% (vol/vol) human plasma (pH 6.5). After gel filtration, ^{3}H and ^{35}S counts were determined for each 2.0-ml fraction in a dual-channel liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.), and ^{125}I and ^{131}I counts were determined for each 0.75-ml fraction in a dual-channel gamma-well spectrometer (Packard Instrument Co.) with appropriate isotope standards to correct for cross-channel interference.

A 20-μl aliquot of unlabeled native bPTH-(1–84), incubated with membranes as described above, was added to 500 μl of 8-M guanidine containing ^{125}I-labeled bPTH-(1–84), ^{125}I-labeled bPTH-(1–34), and Na^{125}I as internal mark-

ers. The latter guanidine mixture was then immediately chromatographed by gel filtration on a 1.2 × 70-cm column of Bio-Gel P-100 at 4°C. The column was eluted with a buffer consisting of 0.05 M barbital (pH 8.5), 0.01 M Na_2EDTA, 0.01% merthiolate, and 2% (vol/vol) human plasma, and 0.9-ml fractions were collected and assayed for ^{125}I radioactivity in a gamma-well spectrometer (Packard Instrument Co.). The fractions (0.1–0.3-ml aliquots in duplicate of each fraction) were then analyzed for PTH by radioimmunoassay with ^{125}I-labeled bPTH and GP-1 antiserum.

Analysis of hormonal form bound to membranes. To assess the nature of the hormone bound to the membrane, 48 μg of bPTH-(1–84) (0.5 μM) was incubated for 10 min at 22°C in a total volume of 10 ml containing 5.0 mg canine membrane protein and the solution described in *Assay of adenylyl cyclase activity* but without [α-^{32}P]ATP, i.e., the usual incubation mixture of 0.5 μM bPTH-(1–84) was increased exactly 100-fold. Simultaneously, 0.5 μM of the same preparation of bPTH-(1–84) was incubated with a 50-μg aliquot of the same membrane preparation for 10 min at 22°C in a total volume of 100 μl containing [α-^{32}P]-ATP and assayed for adenylyl cyclase activity to determine the initial biological activity of the hormone. The large-scale incubation was stopped after 10 min by immediately chilling the incubation mixture to 4°C and centrifuging at 2,200 *g* for 15 min. The supernate was then discarded, and the pellet was resuspended in 10 ml of 0.05 M Tris-HCl buffer, pH 7.4, and recentrifuged at 2,200 *g* for 15 min. The supernate was then removed, and the pellet containing bound hormone or fragment or both was dissolved in either 1 ml of 8 M guanidine or in 1 ml of 8 M urea.

50 μl of each pellet was then removed and appropriately diluted and radioimmunoassayed for PTH, with bPTH-(1–84) as a standard and GP-1 antiserum. An 0.2-ml aliquot of the pellet dissolved in 8 M urea [to which was added ^{131}I-labeled bPTH-(1–84) and ^{125}I-labeled bPTH-(1–34) as markers] was analyzed by electrophoresis on polyacrylamide gels at pH 4.4. 1-mm gel slices were each shaken in 200 μl of a solution of 0.05 M barbital buffer (pH 8.5) with 10% (vol/vol) human plasma for 24 h at 4°C. The resulting samples were counted for radioactivity and analyzed (0.001–0.002-ml aliquots, in duplicate, of each sample) for PTH and fragments by "N" and "C" radioimmunoassays as discussed below. An 0.5-ml aliquot of the pellet suspended in guanidine, to which were added ^{125}I-labeled-bPTH-(1–84), ^{125}I-labeled-bPTH-(1–34), and $Na^{125}I$ as markers, was chromatographed by gel filtration on Bio-Gel P-100 as described in Incubation of hormone with membranes and analysis by gel chromatography. Fractions (0.002–0.015-ml aliquots, in duplicate, of each fraction) were then radioimmunoassayed for PTH and fragments, employing N and C assays.

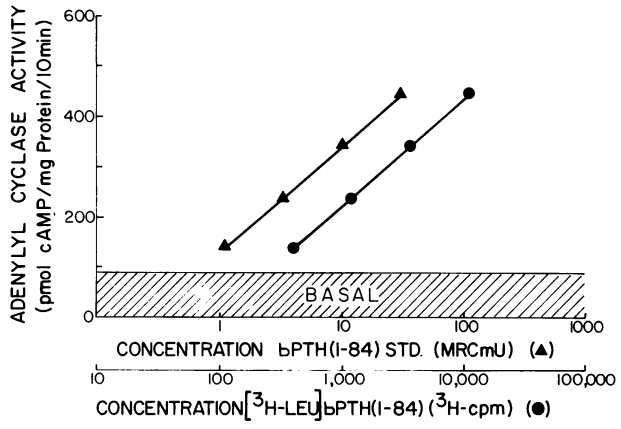

FIGURE 1 Dose-response curve of [^3H]bPTH-(1–84) (●—●) in canine renal cortical membranes. Unlabeled bPTH-(1–84) (▲—▲) (MRC Research Standard 72/286) was used as a standard. Adenylyl cyclase assay was performed at 37°C as described in Methods. Each point represents the mean of duplicate determinations±SE. "Basal" represents adenylyl cyclase activity in the absence of hormone.

NH_2- and COOH-terminal specific radioimmunoassays (N and C assays) used in these studies have previously been described (10). In brief, aliquots of GP-1 antiserum were either preabsorbed with the natural fragment 53–84 and were thus incapable of recognizing the COOH-region of bPTH-(1–84) (N assay) or were preabsorbed with the synthetic fragment bPTH-(1–34) and were thus incapable of recognizing the NH_2-region of bPTH-(1–84) (C assay). These modified antisera, used for measuring samples obtained from gel filtration and gel electrophoresis, were also used to establish appropriate standard curves with bPTH-(1–84) as standard.

A 1.0-ml solution of the pellet in 8 M guanidine [to which were added ^{131}I-labeled bPTH-(1–84), ^{125}I-labeled bPTH-(1–34), and $Na^{125}I$ as markers] was chromatographed by gel filtration on a 1.2 × 70-cm column of Bio-Gel P-30 and eluted with 0.1 M ammonium acetate, pH-6.3, collecting 0.9-ml fractions. The fractions containing the intact hormone peak and the fractions containing the active-fragment peak (as judged from the elution patterns of the marker hormone and marker fragment, respectively) were each pooled separately and lyophilized. Each pool was then dissolved in 50 μl of a solution of 2% BSA—0.0005 N acetic acid, and serial 1:3 dilutions were made. 10 μl, in duplicate, of the three highest concentrations of each pool was then assayed for adenylyl cyclase activity.

Assay of adenylyl cyclase activity. Adenylyl cyclase activity was assayed at 22° or 37°C after 10 min, unless otherwise specified, by a modification (24) of a previously described method (25). The incubation mixture contained, in a 100-μl volume, 50 mM Tris-HCl, pH 7.4; 0.84 mM ATP; 0.8–2.0 × 10^6 cpm of [α-^{32}P]ATP (New England Nuclear Corp., Boston, Mass.); 9 mM theophylline; 4.2 mM $MgCl_2$; 26 mM KCl; 0.118% albumin; 5 mM creatine phosphate (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.); 0.1 mg/ml of creatine phosphokinase; and 30–80 μg of membrane protein. Protein determination was by the method of Lowry et al. (26), with bovine serum albumin as a standard.

RESULTS

Metabolism and biological activity of labeled PTH. The biological activity of biosynthetic [^3H]bPTH-(1–84) was assayed in canine renal cortical membranes at 37°C in four dose dilutions against MRC standard 72/286 (Fig. 1). The hormone was found to have a potency of 2.7 mU/1,000 cpm. This facilitated the use of a concentration of hormone for incubation studies that was no greater than half-maximal (27 mU) but still represented sufficient radioactivity (up to 10,000 cpm) for fragment detection.

After [^{3}H]bPTH-(1–84) was incubated with active canine renal cortical membranes for 10 min at 22°C, more than 95% of the ^{3}H radioactivity was precipitable with 15% trichloroacetic acid. Analysis by electrophoresis on polyacrylamide gels revealed only a single peak of radioactivity, containing 65% of the added counts, that comigrated with the [^{14}C]bPTH-(1–84) marker (Fig. 2A); less than 3% of the added counts overlapped into the region corresponding to the migration position of bPTH-(1–34), and no peak appeared in this region of the gel. Furthermore, these results were the same as those obtained by incubation of [^{3}H]bPTH-(1–84) with heat-inactivated membranes used as a control.

When [^{3}H]bPTH-(1–84) was incubated with active canine renal cortical membranes for 10 min at 22°C and analyzed by chromatography on Bio-Gel A in 6 M guanidine hydrochloride, only a single peak of radioactivity containing 60% of added ^{3}H radioactivity, and coeluting with the [^{35}S]bPTH-(1–84) marker, appeared (Fig. 2B). No other peaks were seen, and less than 2% of added ^{3}H radioactivity spilled over into the fractions corresponding to the elution position of the hormone fragment. These results were identical with those obtained (Fig. not shown) by incubating the hormone with heat-inactivated membranes as a control.

Analysis by gel filtration in 6 M guanidine hydrochloride of a mixture of ^{125}I-labeled bPTH-(1–84) and active canine membranes incubated for 10 min at 22°C also revealed only a single peak of radioactivity coeluting with the ^{125}I-labeled bPTH-(1–84) marker (Fig. 3A). The pattern of eluted radioactivity was essentially indistinguishable from that obtained after incubating the hormone with heat-inactivated membranes (Fig. 3B). Although more than 83% of the ^{125}I-radioactivity added to the membranes was recovered in the column eluate, less than 0.8% (less than 3 pg) was recovered in the eluate corresponding to the elution position of bPTH-(1–34).

Metabolism and biological activity of unlabeled PTH. When unlabeled native bPTH-(1–84), 0.5 μM, was incubated with active canine renal membranes for 10 min at 22°C and a 96-ng aliquot was then analyzed by electrophoresis on polyacrylamide gels and radioimmunoassay, only a single peak of immunoreactive material was seen, comprising 47% of the immunoreactivity added and comigrating with the [^{125}I]-labeled-bPTH-(1–84) marker (Fig. 4A). No immunoreactive peak was seen comigrating with the fragment marker, and less than 3% of the immunoreactivity eluted in this region. This was identical with the electrophoretic profile obtained after incubation with heat-inactivated membranes (Fig. 4A).

When another 96-ng aliquot of an identical solution of unlabeled bPTH-(1–84) and active canine membranes, incubated simultaneously with the previous mixture, was analyzed by gel filtration on Bio-Gel P-100 and by radioimmunoassay, the immunoreactive material recovered (65% of the added hormone) coeluted with the ^{125}I-labeled-bPTH-(1–84) marker (Fig. 4B). No immunoreactive peak was seen coeluting with the fragment marker, and no more than 2% of the added hormone could have eluted here and escaped detection by the radioimmunoassay. Furthermore, the chromato-

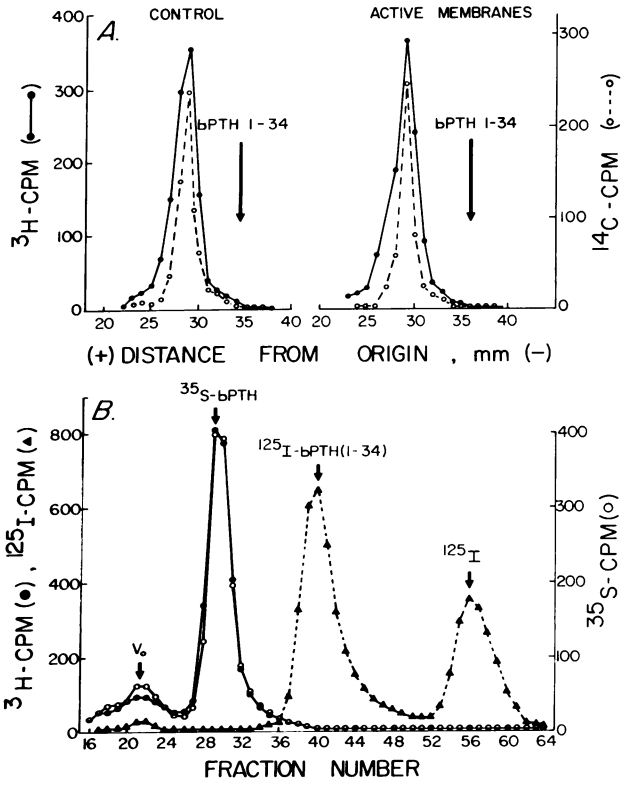

FIGURE 2 Analysis of [^{3}H]bPTH-(1–84), after incubation with canine renal cortical membranes for 10 min at 22°C, by electrophoresis (Fig. 2A) on polyacrylamide gels at pH 4.4, and by chromatography (Fig. 2B) on Bio-Gel A-0.5m with 6 M guanidine HCl–0.1 M ammonium acetate–1% (vol/vol) human plasma, pH 6.5, as eluting buffer. For electrophoresis (Fig. 2A), [^{14}C]bPTH-(1–84) was added to the gels to serve as an internal marker for intact hormone. The arrow (Fig. 2A) represents the migration position of ^{125}I-labeled bPTH-(1–34) run in parallel gels. "Control" (Fig. 2A) represents the pattern obtained after incubation of [^{3}H]bPTH-(1–84) with heat-inactivated membranes. For chromatography (Fig. 2B), [^{35}S]bPTH-(1–84) was added to the column to serve as an internal marker for intact hormone. The column was subsequently calibrated with ^{125}I-labeled bPTH-(1–34) and Na^{125}I to mark the elution positions of active fragment and salt peak, respectively. V_o represents the void volume. A pattern of radioactivity identical with Fig. 2B was obtained after incubation of [^{3}H]bPTH-(1–84) with heat-inactivated membranes. Details of the incubation, electrophoresis, and chromatography are described in the text.

graphic profile was identical with the pattern obtained (Fig. not shown) after incubation with heat-inactivated membranes.

The adenylyl cyclase activity of the remainder of the incubation mixtures from which aliquots had been removed for electrophoresis and chromatography is demonstrated in bars A and C in the inset in Fig. 4. There was simultaneous determination of the activity of incubation mixtures containing either active membranes but no active hormone (bar B), or active membranes with active hormone from which no aliquots had been removed (bar D). These experiments demonstrated the presence of biological activity in the membranes at 22°C and the adequacy of the heat-inactivated membranes as a control. More important, they demonstrated the pres-

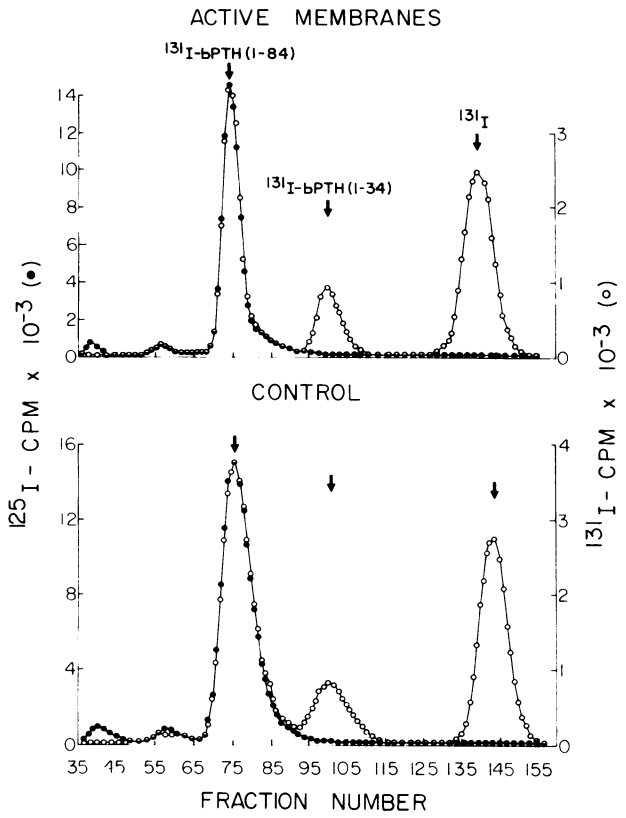

FIGURE 3 Gel chromatography profiles of radioactivity after incubation of ^{125}I-labeled bPTH-(1–84) with canine renal cortical membranes for 10 min at 22°C. Incubation conditions are described in detail in the text. Chromatography was on Bio-Gel A-0.5m with 6 M guanidine HCl-0.1 M ammonium acetate–1% (vol/vol) human plasma, pH 6.5, as eluting buffer. Details of chromatography are described in the text. The arrows, from left to right, represent the elution positions of ^{131}I-labeled bPTH-(1–84), ^{131}I-labeled bPTH-(1–34), and Na^{131}I, respectively, which were added to the columns immediately before chromatography to serve as markers. "Control" represents the pattern obtained after incubation of ^{125}I-labeled bPTH-(1–84) with heat-inactivated membranes.

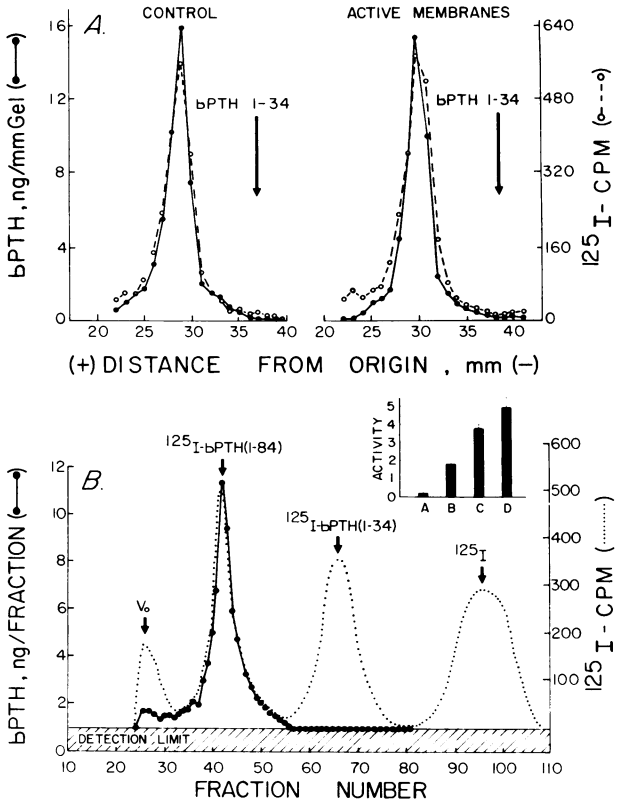

FIGURE 4 Analysis of unlabeled immunoreactive bPTH-(1–84), after incubation with canine renal cortical membranes for 10 min at 22°C, by electrophoresis (Fig. 4A) on polyacrylamide gels at pH 4.4, by chromatography (Fig. 4B) on Bio-Gel P-100, and by bioassay (inset). For electrophoresis (Fig. 4A), ^{125}I-labeled bPTH-(1–84) (^{125}I pattern of migration shown) and ^{125}I-labeled bPTH-(1–34) (migration position represented by the arrow) were added to the gels to serve as internal markers for intact hormone and active fragment, respectively. "Control" represents the electrophoretic profile obtained after incubation of unlabeled bPTH-(1–84) with heat-inactivated membranes. For chromatography (Fig. 4B), ^{125}I-labeled bPTH-(1–84), ^{125}I-labeled bPTH-(1–34), and Na^{125}I were added to the column to serve as internal markers. V_o represents the void volume. An identical elution pattern was obtained after incubation of unlabeled bPTH-(1–84) with heat-inactivated membranes. Details of the incubation, electrophoresis, chromatography, and immunoassays with nonadsorbed GP-1 antiserum are described in the text. Assay of adenylyl cyclase activity of samples simultaneously analyzed by electrophoresis and chromatography is depicted in the inset. "Activity" represents adenylyl cyclase activity (pmol cAMP[$\times 10^{-2}$]/mg protein/10 min). Bar A represents the adenylyl cyclase activity of heat-inactivated membranes. Bar B represents the enzyme activity of the membranes in the absence of hormonal addition (nonstimulated or basal). Bar C represents the adenylyl cyclase activity stimulated by 0.5 μM bPTH-(1–84) from which 20 μl (20%) of the incubation volume was removed for electrophoresis before assay. Bar D represents the adenylyl cyclase activity stimulated by 0.5 μM bPTH-(1–84) from which there were no removals before assay. The assays were performed as described in the text. Each bar represents the mean of triplicate determinations±SE.

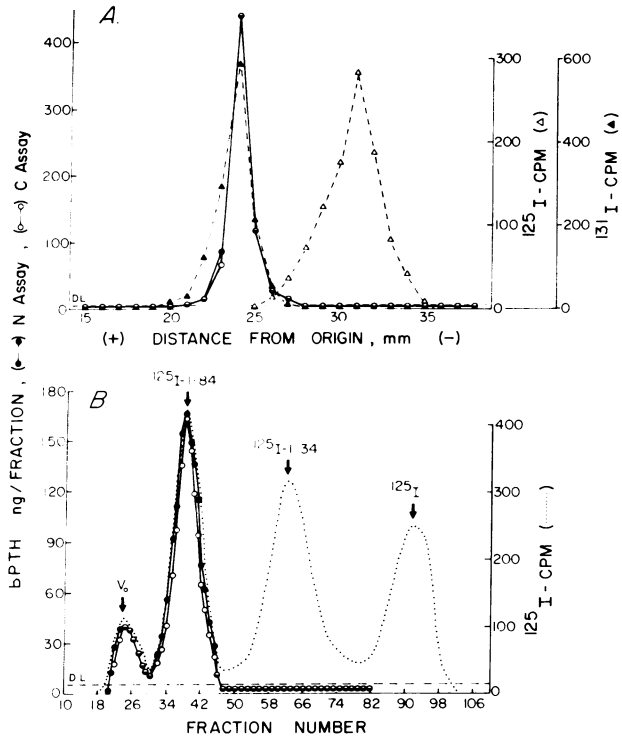

FIGURE 5 Analysis of membrane-bound unlabeled bPTH-(1–84) incubated with active canine renal cortical membranes for 10 min at 22°C. The membrane-bound hormone was solubilized as described in the text and analyzed by polyacrylamide gel electrophoresis at pH 4.4 (Fig. 5A) or by gel filtration on columns of Bio-Gel P-100 (Fig. 5B). Hormone in each gel slice (Fig. 5A) or each column fraction (Fig. 5B) was immunoassayed with separate N and C assays as described in the text. ^{131}I-labeled or ^{125}I-labeled bPTH-(1–84), ^{125}I-labeled bPTH-(1–34), and Na^{125}I (for gel filtration only) were added before electrophoresis or chromatography to serve as internal markers. V_o represents the void volume of the column, and DL the detection limit of the immunoassay. Details of the experimental procedures are described in the Methods section.

ence of biological activity in the native PTH simultaneously subjected to analysis by electrophoresis and chromatography.

Metabolism and biological activity of membrane-bound PTH. To ascertain that no fragmentation of the hormone was being detected in the incubation mixture because only a small fraction of the total hormone, (i.e., that fraction bound to membrane receptors) was being metabolized, the experimental design was modified, as described in Methods, to permit chemical and biological characterization of the hormone bound to membrane.

The unlabeled hormone bound to the active canine membrane represented 7.3% (mean of triplicate determinations) of the unlabeled bPTH-(1–84) added to the incubation mixture, as determined by radioimmunoassay.

When analyzed by polyacrylamide gel electrophoresis and radioimmunoassay employing region-specific N and C assays, the hormone bound to the membrane migrated as a single peak, comigrating with the ^{131}I-labeled bPTH-(1–84) intact hormone marker and representing 89% of the added immunoreactivity (Fig. 5A). No divergence of N and C immunoreactivity was seen, and no peak was seen migrating in the region of the ^{125}I-labeled bPTH-(1–34) fragment marker. No more than 1% of the NH_2-terminal immunoreactivity in the pellet could have migrated in the eight slices comprising the hormone fragment peak and escaped detection by the radioimmunoassay. Inasmuch as the hormone bound to the pellet comprised approximately 7% of the total hormone added to the incubation mixture, the maximum NH_2-terminal immunoreactivity bound to the membrane that could have escaped detection is 0.07% of the hormone added to the incubation mixtures.

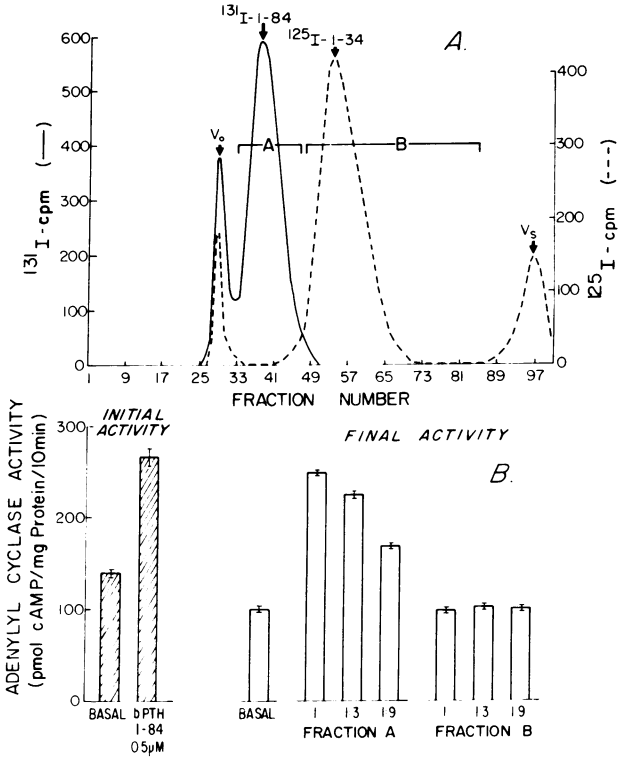

FIGURE 6 Adenylyl cyclase activity relative to basal, of bPTH-(1–84) before (Initial Activity) and after (Final Activity) incubation with active canine renal cortical membranes for 10 min at 22°C (Fig. 6B). Each bar (Fig. 6B) is the mean of triplicate determinations±SE. The final activity assayed in three dilutions was the activity of membrane-bound peptide pooled from fractions eluting from a column of Bio-Gel P-30 (Fig. 6A) either with the intact hormone marker, ^{131}I-labeled bPTH-(1–84) (Fraction A), or later than the intact hormone but before the salt peak (Fraction B). V_o and V_s represent the void volume and salt peak of the column, respectively. Details of the procedures are described in the text.

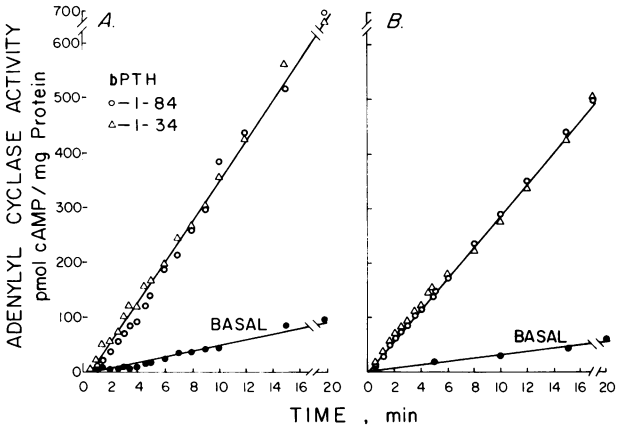

FIGURE 7 Generation of cAMP in the absence of added hormone ("basal") in the presence of 0.02 μM (Fig. 7A) or 0.5 μM (Fig. 7B) bPTH-(1–34), and in the presence of 0.02 μM (Fig. 7A) or 0.5 μM (Fig. 7B) bPTH-(1–84). Canine (Fig. 7A) or rat (Fig. 7B) renal cortical membranes (0.5 mg/ml) were incubated for adenylyl cyclase activity at 22°C with the appropriate concentrations of hormones in a final incubation volume of 2.3 ml. Generation of cAMP was followed by removing, at the times indicated, 100-μl aliquots and determining the [^{32}P]cAMP formed from [α-^{32}P]ATP as described in the text. The remainder of the experimental conditions is given in Methods.

When analyzed by gel filtration and radioimmunoassay employing N and C assays, the membrane-bound hormone coeluted with the ^{125}I-labeled bPTH-(1–84) intact hormone marker (62% of the added immunoreactivity was recovered in the intact hormone peak) (Fig. 5B). No immunoreactivity coeluted with the ^{125}I-labeled bPTH-(1–34) fragment marker, and no more than 3% of the NH_2-terminal immunoreactivity in the pellet could have eluted in the 10 fractions comprising the hormone fragment elution position and escaped detection by radioimmunoassay. Consequently, the maximum NH_2-terminal immunoreactivity bound to the membrane that could have escaped detection by this method is 0.2% of the hormone added to the incubation mixture.

To ascertain whether the minute amounts of immunoreactivity that might have escaped detection by the methods employed were indeed of biological significance, the membrane-bound hormone was again fractionated by gel filtration. The eluate fractions corresponding to (*a*) the intact hormone and (*b*) any possible hormone fragments smaller than the intact hormone but larger than ^{125}I were pooled separately (Fig. 6). Each fraction was tested for biological activity in canine membranes in three dilutions (Fig. 6). Only fraction *a* demonstrated the capacity to stimulate adenylyl cyclase. Fraction *b* exhibited no stimulation of adenylyl cyclase activity above basal.

Kinetics of hormone-stimulated adenylyl cyclase. The kinetics of hormone-stimulated adenylyl cyclase were studied to assess the possible relationship, with regard to biological activity, between bPTH-(1–84) and an active amino-terminal fragment, as precursor and product, respectively. The generation of cyclic 3′,5′-adenosine monophosphate (cAMP) with time was determined in canine and rat renal cortical membranes at 22°C in the absence of hormone and in the presence of equimolar submaximal concentrations of bPTH-(1–84) and bPTH-(1–34), 2×10^{-8} M in the canine and 10^{-6} M in the rat membranes, respectively (Fig. 7). The generation of cAMP was linear over 20 min in both membrane systems, and bPTH-(1–84) and bPTH-(1–34) demonstrated virtually superimposable curves of adenylyl cyclase activity during this time in both membrane systems.

Sensitivity of adenylyl cyclase assay. The electrophoretic and chromatographic profiles of [^{3}H]bPTH-(1–84) were compared after incubation with active and heat-inactivated bovine, rat, and canine renal cortical membranes for 10 min at 37°C. With canine membranes, the profiles obtained after incubation at 37°C were identical with those seen after incubation at 22°C (Fig. 2).

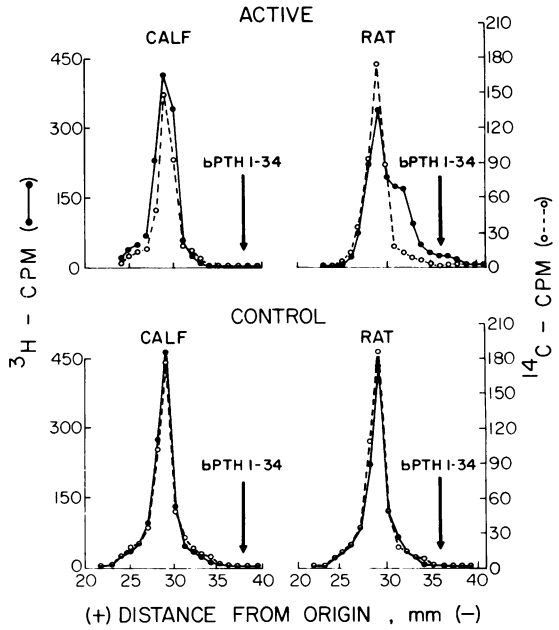

FIGURE 8 Electrophoretic profiles of radioactivity on polyacrylamide gels at pH 4.4 after incubation of [^{3}H]bPTH-(1–84) with calf or rat renal cortical membranes for 10 min at 37°C. Upper profiles represent incubations performed with active membranes, and lower profiles, incubations with heat-inactivated membranes used as controls. [^{14}C]bPTH-(1–84) was added to the gels to serve as an internal marker for the intact hormone. The arrows represent the migration positions of ^{125}I-labeled bPTH-(1–34) run in parallel gels. Details of the incubation and electrophoretic analysis are described in the text.

Only active rat membranes produced significant degradation of bPTH-(1–84) (Figs. 8 and 9).

The adenylyl cyclase activities of the three types of membranes in the presence of increasing concentrations of bPTH-(1–84) were then compared (Fig. 10). Half-maximal activation of bovine and canine adenylyl cyclase was achieved at concentrations of 10^{-8} M and 1.5×10^{-8} bPTH-(1–84), respectively. Adenylyl cyclase prepared from rat membranes was much less sensitive, requiring 5×10^{-7} M bPTH-(1–84) for half-maximal activation.

DISCUSSION

Previous demonstration that PTH, added as an intact peptide of 84 amino acids directly to suspensions of renal cortical membranes, stimulated adenylyl cyclase (*27*) apparently established that the intact polypeptide was indeed active without the requirement for metabolic alteration before receptor interaction. Evidence has accumulated from multiple laboratories, however, concerning generation of hormonal fragments (*7–14*), of which some, on the basis of the region of the hormone sequence they represented and knowledge of structure-activity relationships (5), must be considered as potentially biologically active (5). The exact tissue site of proteolysis is unclear (*8, 12, 13, 15, 17, 18, 20*); however, in addition to possible cleavages within the parathyroid glands or in peripheral tissues generally, cleavage in sites adjacent to receptors in target organs (*19, 28, 29*) must be considered. This has led several laboratories to the logical conclusion that, if proteolytic enzymes were active in renal cortical membrane suspensions used for assays in vitro, cleavage of the intact hormone into active fragments might occur, and hence the fragments, rather than intact hormone, might be the active species (15, 18, 20). The present studies were therefore undertaken to examine critically whether cleavage of hormone into fragments occurred on the renal cortical membrane suspensions where adenylyl cyclase, the marker of biological activity, is stimulated in vitro.

Two labeled preparations of hormone, biosynthetic, internally labeled [^{3}H]bPTH-(1–84) and ^{125}I-labeled bPTH-(1–84), were studied in addition to preparations of native biologically active unlabeled bPTH-(1–84). Metabolism of these hormones in renal membranes was studied at two temperatures, 22° and 37°C, in preparations of kidney tissue from three species, rat, dog, and cow. The integrity of the hormone after incubation with renal membranes was assessed on the basis of both charge and size by electrophoresis and gel filtration with and without added denaturing solvents. The analytic systems used achieve excellent resolution of intact hormone from hormonal fragments (Fig. 5). No chemical or immunochemical evidence of hormonal degradation

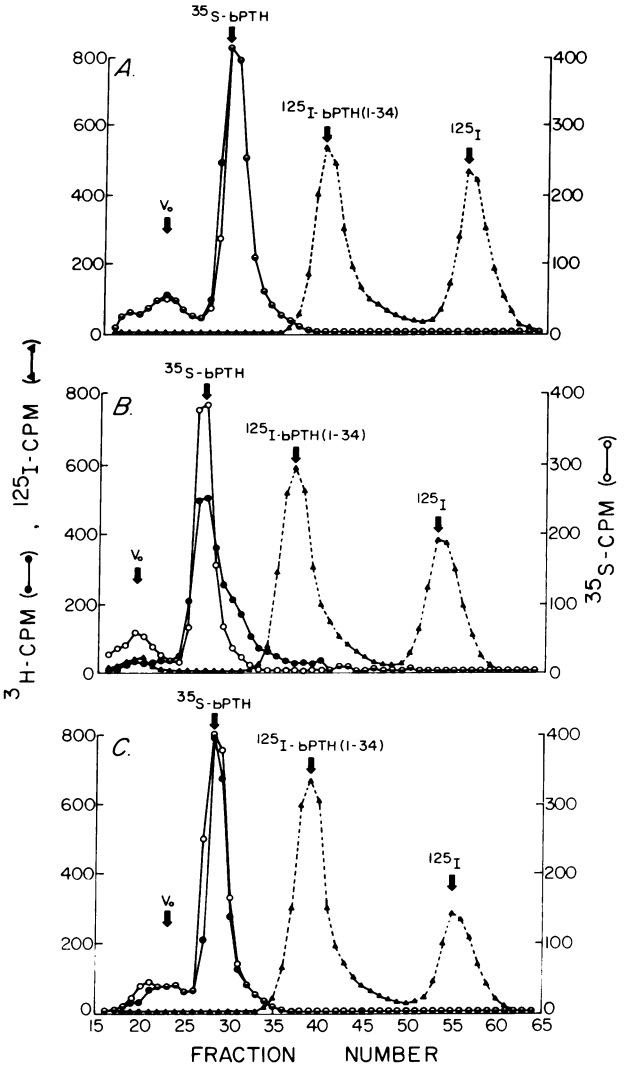

FIGURE 9 Elution profiles of radioactivity from columns of Bio-Gel A-0.5m after incubation of [^{3}H]bPTH-(1–84) with active calf (Fig. 9A), active rat (Fig. 9B), or inactive rat (Fig. 9C) renal cortical membranes for 10 min at 37°C. [^{35}S]bPTH-(1–84) was added to the columns just before chromatography to serve as a marker for intact hormone. The columns were subsequently calibrated with ^{125}I-labeled bPTH-(1–34) and Na^{125}I to mark the elution positions of active fragment and salt peak, respectively. V_o represents the void volume. The pattern obtained after incubation of [^{3}H]bPTH-(1–84) with heat-inactivated calf membranes for 10 min at 37°C was identical with the pattern in 9A. Details of the incubation and chromatography are described in the text.

could be demonstrated during an interval of membrane exposure associated with strong adenylyl cyclase stimulation in bovine and canine membranes. Furthermore, when stimulation of adenylyl cyclase by bPTH-(1–84) and bPTH-(1–34), respectively, was studied as a function of time, no significant difference was seen in the

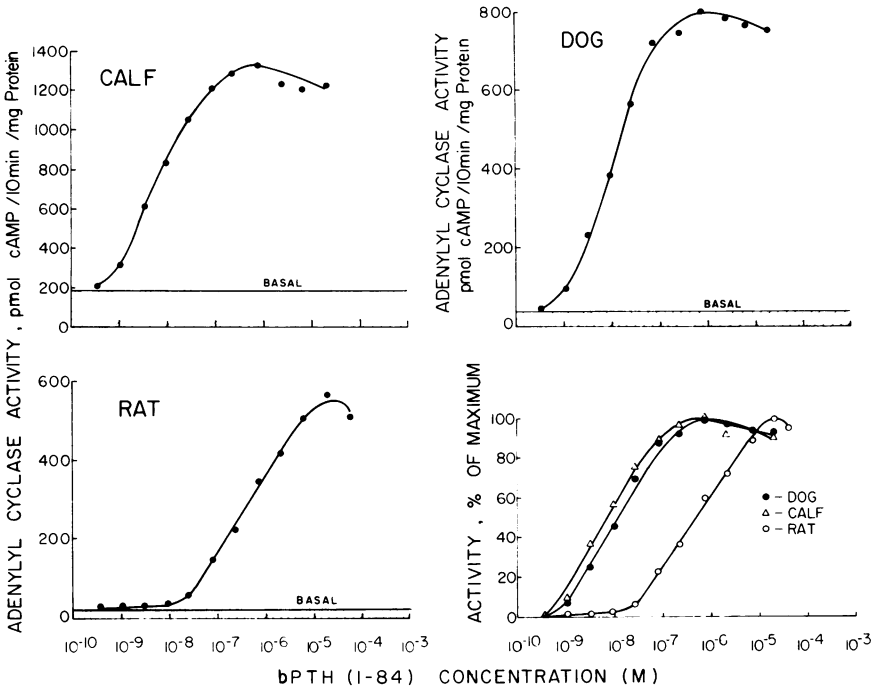

FIGURE 10 Adenylyl cyclase activity of renal cortical-membrane preparations from calf (upper left), dog (upper right), and rat (lower left) stimulated by increasing concentrations of bPTH-(1–84). Hatched area represents the basal adenylyl cyclase activity. Each point is the mean of triplicate determinations; individual determinations varied by less than 10% from the mean. At the lower right are depicted the relative sensitivities of these membranes, in which adenylyl cyclase activity, stimulated by increasing concentrations of bPTH-(1–84) in membranes from each species, is expressed as percent of maximum hormone-stimulated activity, corrected for basal activity. Assay of adenylyl cyclase activity was performed as described in the text after a 10-min incubation at 37°C.

time-course of the stimulation produced; that is, there was no significant lag before the onset of stimulation by bPTH-(1–84) in comparison with bPTH-(1–34) in either dog or rat membranes, and the rates of formation of cAMP were identical over the 20-min interval for both intact hormone and active amino-terminal fragment in both species. Consequently, the kinetics of hormone-stimulated adenylyl cyclase activity do not support the concept that a precursor-product relationship between bPTH-(1–84) and amino-terminal fragments is necessary for adenylyl cyclase stimulation.

When the issue of hormonal degradation was examined at 37°C with the three membrane preparations employed, only purified membranes from the rat showed any degradative activity. This is consistent with previous reports of degradation of bPTH by rat microsomal fractions of rat homogenates (30–32). When the degradation of bPTH is analyzed, however, in conjunction with the dose-response curves, it appears that this proteolysis, rather than being important in the activation of PTH, may contribute to the decreased sensitivity of the adenylyl cyclase assay performed with rat kidney membranes. This concept is supported by the finding of identical kinetics of activation of adenylyl cyclase by both the hormone and active fragment, even in rat membranes.

Not only are these overall findings consistent with the thesis that proteolytic modification is not required before stimulation of adenylyl cyclase by intact PTH, but the results may also be of practical interest in the design of bioassays in vitro for the hormone. Thus, detailed features of structure-activity relationships such as receptor-binding requirements (and inhibition of binding by antagonists and partial agonists [24]), as well as analysis of cofactors and essential steps in hormone action in vitro, may be made more easily in membrane systems from dog and cow, which exhibit a greater sensitivity and a lesser degree of contamination with degradative enzymes than do the previously described and widely used membrane preparations from rats.

In considering the major issue addressed in these studies, namely, whether intact hormone does act on receptors in bone and kidney in vivo without prior cleavage, several potential problems must be considered with

regard to interpretation of the results. These issues include the following: (*a*) whether the hormone preparations used are biologically active and/or adequately reflect the metabolic fate on membranes of endogenously secreted hormone; (*b*) whether cleavage, if it occurs before activation of adenylyl cyclase, could have been detected by the methods employed; (*c*) whether the adenylyl cyclase assay in vitro as presently used is an adequate model for analysis of essential features of hormone action in vivo.

With regard to the first issue, i.e., the adequacy of the types of hormone preparations used as markers for the metabolic fate of endogenous hormone, considerable reassurance is provided by the variety of hormone preparations used. Although ^{125}I-labeled PTH may be biologically inactive as a result of oxidation of methionine residues during iodination (33), in view of its high specific activity, it provides the considerable advantage of ease of detection of small concentrations of generated fragments. Furthermore, ^{125}I-labeled PTH, when injected into dogs, is cleaved in a precise fashion that, by gel filtration, is indistinguishable from the cleavage pattern generated in vivo when unlabeled biologically active hormone is used in identical studies (15). Hence, we can state that the very cleavage process seen in vivo that has led us to investigate whether cleavage precedes hormone action can be detected with ^{125}I-labeled PTH. Therefore, it seems appropriate to use the ^{125}I-labeled PTH as a probe for cleavage by renal cell membrane suspensions. Because the issue of cleavage before action is so critical in many aspects of PTH physiology, it seemed essential to use other preparations of hormone known to be biologically active. The criterion of known biological activity could be met with the tritiated hormone preparations. Incorporation of tritiated amino acids per se does not cause loss of activity, and the preparation after internal labeling was purified with special precautions to ensure against loss of activity during gel electrophoresis. The potency of the tritiated hormone preparation was directly determined by bioassay (Fig. 1), which confirmed that it was biologically active. As a final precaution, and to further increase the confidence limits for detection of cleavages, we used unlabeled hormone, previously demonstrated to possess full specific biological activity, to provide an independent third approach to study the cleavage problem by immunological techniques during fractionation studies after exposure of the hormone to membranes.

With regard to the second issue, each of the hormone preparations was examined in different mammalian species. Hormones were added to membrane suspensions in concentrations well below those required to elicit maximal adenylyl cyclase responses to eliminate the danger of supersaturation of either adenylyl cyclase-linked receptors or cleavage enzymes. Hormone integrity was assessed by two separate methods, electrophoresis and gel filtration, each suitable for identifying fragmentation on a different physiochemical basis, with resolving power and sensitivity sufficient for the detection of as little as 3% cleavage of the hormone added to the incubation mixture. To further increase the sensitivity of detection of any cleavage and to directly study that fraction of the hormone presumed to activate hormone receptors, we studied membrane-bound hormone rather than hormone recovered from the entire membrane suspension. Chemical and immunochemical analyses of the membrane-bound hormone reduced by another order of magnitude any possibility of failure to detect cleavage into an active fragment. By one method as little as 0.2% and by another method as little as 0.07% of hormone added to the incubation mixture could have been detected if cleaved. If the total potency of the native, unlabeled bPTH-(1–84) (3,000 U/mg) resided in a cleavage product, i.e., an active fragment present at concentrations beyond the detection limit of the methods used, the potency of such a putative active fragment can be calculated and, in turn, searched for by bioassay. The calculated potency, based on the estimate that less than 0.2–0.07% of the added hormone is active, is seen to be 1.5–4.3-million U/mg, a potency far beyond that of any fragment yet synthesized or isolated (5). These considerations led to yet another approach. To determine whether biological activity did indeed reside in the portion of the column eluate in which no fragment or hormone could be detected by immunochemical means, this fraction of the column eluate was pooled and bioassayed. No biological activity was seen in this fraction, whereas activity could be readily demonstrated in the portion of the eluate corresponding to the elution position of intact hormone.

The present findings are not inconsistent with the findings of others (19, 28) of metabolism of PTH by whole canine kidney or by canine renal membranes in vitro. It is not inconceivable that with sufficient duration of exposure, or with a very large increase in the ratio of membrane to hormone (and therefore an increase in the ratio of potential proteolytic enzymes to substrate), or with the use of other membranes from other anatomical portions of the renal cell, metabolism of PTH could have been demonstrated. The object of these studies, however, was to determine the necessity of hormone metabolism for biological action and not the capacity of renal membranes to metabolize PTH. Consequently, conditions of temperature and membrane purification were chosen to achieve the minimum proteolysis possible while still demonstrating significant biological activity. These efforts have indicated that biological action may occur in the absence of proteolysis.

With regard to the third issue, if analysis of cleavage in relation to biological activity of PTH in these membranes is to serve as a valid model for understanding the nature of the active molecular species involved in biological action of the hormone in vivo, then it must be ascertained that stimulation in vitro of adenylyl cyclase in renal cortical-cell membranes faithfully reflects specificity and binding affinity for receptors in vivo. The observation that intact hormone acts without prior cleavage on the renal receptor for hormonally sensitive adenylyl cyclase is of general significance for hormonal interaction with physiological receptors in kidney and bone in vivo only in so far as the requirements for activation in the adenylyl cyclase assay are the same as requirements for hormonal effects on calcium and phosphate transport in vivo.

With regard to specificity, structure-function studies with bPTH and numerous analogues have demonstrated, in general, good correlation between the activity of the analogues in vitro in the renal adenylyl cyclase assay in the rat, and in vivo in various assays including hypercalcemia in the chick (believed to reflect primarily action on bone) (5) and urinary excretion of phosphate and cAMP in the rat (34, 35). There are residual questions, however, about binding affinity. In particular, the assay in vitro appears to be much more influenced by alterations in the amino-terminal residue of PTH (position 1) than does the assay in vivo (35). The discrepancy between results in vivo and in vitro exhibited by analogues with alterations at position 1 are not explicable merely by invoking different structural requirements for bone receptors and renal receptors, because these forms of the hormone are equipotent with the bovine 1–84 standard when assayed for urinary cAMP (34) or phosphate excretion (35) in vivo in rats. Furthermore, an extreme divergence is noted between dose-responses of intact hormone in vitro (even in the more sensitive dog and cow membrane system) (100 nM) and those detected in vivo (10 pM) (35).

One could explain some of these discrepancies in results of bioassays in vivo and in vitro by the view that much of the binding in vitro represents the effect of high-capacity, low-affinity binding sites (requiring hormone concentrations of 100 nM) that differ in binding requirements from those of the presumed high-affinity receptors coupled to physiological responses, such as phosphaturia or calcium mobilization in vivo (stimulated at concentrations of 10 pM). Overall, however, the abundant accumulated evidence that generation of cAMP is a critical intermediate in expression of PTH action in vivo (34) and the good correspondence in structure-activity studies with hormone analogues between the in vitro adenylyl cyclase assay and in vivo assays suggest that the in vitro system employed here is an appropriate test system.

Hence, despite the reservations concerning the adequacy of the in vitro assay as a model for hormone action in vivo, the present results do provide considerable data, involving multiple hormone preparations, membrane systems, and analytical methods, hitherto not available, that cleavage of the intact hormone seems not to be required for expression of activity, at least in renal receptors.

These observations in vitro should not be considered to disagree with findings and conclusions of others or ourselves concerning the existence of fragments in the circulation or the peripheral metabolism, or both, of hormone. Although the present studies support the concept that cleavage of PTH is not required before its action, they do not diminish the potential physiological significance of the sensitive and specific metabolic pathway of the hormone detected in recent studies (15–18), or the importance of circulating fragments detected by still other groups (11–13). Thus, it may be proved ultimately that metabolism of PTH results in production of fragments that are biologically significant with a spectrum or duration of activity that differs from those of the intact hormone, even though intact hormone itself does act directly on receptors. Alternatively, the degradation of hormone may play a physiologically important catabolic function, namely, acceleration of the removal of biologically active hormone from blood. Peripheral metabolism could prove to be an important control point for the overall regulation of the action of PTH. Such issues can only be resolved with further studies now in progress in several laboratories.

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