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Calcium-Regulated Parathyroid Hormone Peptidase

(pig/EDTA/prohormone/precursor/bovine hormone)

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ABSTRACT **An enzymatic activity that catalyzes the conversion of glandular parathyroid hormone to a smaller-molecular-weight, biologically and immunologically active form of this hormone has been extracted from normal parathyroid and other porcine tissues. The product of the conversion has the immunologic characteristics of the hormone that occurs in peripheral serum and in tissue culture medium from parathyroid explants. The enzyme is activated by chelating agents and is inhibited by high calcium concentrations, suggesting that this enzyme may be important in the regulation of secretion or metabolism of the hormone.**

The parathyroid hormone (PTH) extracted from parathyroid tissue is immunologically different from the PTH present in peripheral serum (1–3) and from the PTH released into the medium of cultured parathyroid tissues (3–6). Furthermore, the PTH in urea extracts of parathyroid tissue (3, 5), in the medium of cultured parathyroid tissue explants (4, 5), and in serum (1, 2)† is immunoheterogeneous. The immunologically distinct PTH components in these materials differ in molecular weight as assessed by gel filtration techniques, and those in serum appear to differ in their half-lives of circulation (2–5).† The physiologic importance of these phenomena is unknown. However, they must be integrated into any proposed model of the biosynthesis, secretion, and peripheral metabolism of PTH.

Sherwood and coworkers (5) reported that ^{131}I-bPTH was converted into a smaller-molecular-weight component when incubated in a homogenate of bovine parathyroid tissue. This component coeluted, on gel filtration, with the most prominent immunoreactive species of PTH obtained from the medium of cultured bovine parathyroid explants.

The present report describes an enzymatic activity in crude saline extracts of porcine parathyroid glands and other tissues that converts the PTH in these extracts or added bPTH into a smaller-molecular-weight, biologically active species that is immunologically similar to the PTH in peripheral serum. This PTH peptidase activity is stimulated by the absence of calcium (presence of EDTA or EGTA) and is inhibited by high ambient calcium concentrations. These observations suggest the possibility that a calcium-regulated enzymatic conversion of PTH from its glandular to its predominant circulating form may be a control mechanism in the metabolism of PTH.

Abbreviations: PTH, parathyroid hormone; pPTH, porcine parathyroid hormone; bPTH, bovine parathyroid hormone; ^{131}I-bPTH, ^{131}I-labeled bovine parathyroid hormone; EGTA, ethylenebisoxyethylenenitriolotetraacetate.

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† Canterbury, J. M. & Reiss, E. (1971) "Fractionation of circulating parathyroid hormone (PTH) in man," *J. Lab. Clin. Med.* **78**, 814 abstr.

MATERIALS AND METHODS

Bovine PTH was prepared by the method of Hawker and associates (7) and was 95% homogeneous on the basis of disc gel electrophoresis and amino-acid composition (7–9). It was labeled with ^{131}I (Union Carbide) to specific activities of 300–400 Ci/g, according to the Berson and Yalow (10) modification of the Hunter and Greenwood method (11). At the time of use, the labeled hormone was free of ^{131}I and "damaged" components, as assessed by hydrodynamic flow chromatoelectrophoresis (12). Porcine PTH was prepared by the method of Littledike and Hawker (13) and was about 50% homogeneous.

Radioimmunoassay of PTH was performed as described (14), with an antiserum (GP 1M) against pPTH in a final dilution of 1:75,000. This antiserum, as well as another (CH 14M, anti-bPTH) used in ^{131}I-bPTH binding experiments, distinguishes between the glandular form of PTH and the PTH in human hyperparathyroid peripheral serum or in the medium of cultured parathyroid explants (3, 4, 6). Radioimmunoassay curves obtained with various PTH preparations were fitted for comparison over the same dilution range (1, 3, 4).

Porcine tissues were obtained at an abattoir. Parathyroids were identified as described by Littledike (15). A 10% tissue homogenate was prepared at 4° in 0.9% NaCl with ground-glass homogenizers. The homogenate was centrifuged at 4° for 15 min at 12,500 × *g* in a Sorvall RC2B centrifuge. Aliquots (0.6 ml) of the supernatant fraction were incubated in 12 × 75-mm flint-glass test tubes in a shaking water bath at 37°. Calcium chloride, EDTA, or EGTA from 10-times concentrated stock solutions (pH 7.4) were added to the incubation mixtures to final concentrations of 1.0 and 5.0 mM. The pH of the incubation mixture was maintained in all experiments with 0.1 M imidazole buffer. In experiments in which ^{131}I-labeled bPTH was used, bovine serum albumin, 1 mg/ml, was added as a protein carrier. Trypsin inhibitor and iodoacetate (Sigma) were used at the concentrations indicated below.

Gel filtration of the extracts was performed on 50 by 1 cm (flow rate, 65 μl/min) and 100 by 1 cm (flow rate, 165 μl/min) columns of Sephadex G-50 fine (Pharmacia) in ammonium acetate buffer (0.2 M, pH 4.7)–bovine serum albumin (1 mg/ml).

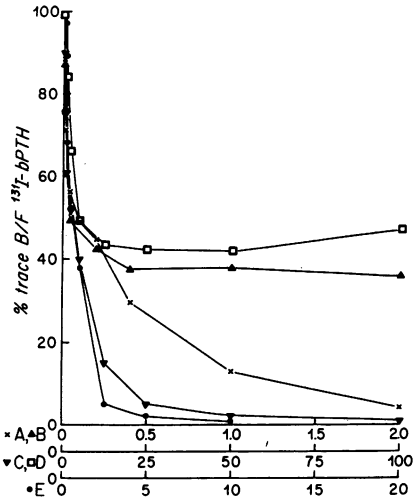

FIG. 1. Immunologic characteristics of PTH preparations in radioimmunoassay with GP 1M anti-PTH antiserum (1:-75,000). Curve *A*: saline parathyroid extract before incubation (μl/ml). Curve *B*: saline parathyroid extract after incubation for 6 hr in the presence of 5 mM EDTA at pH 6.0 and 37° (μl/ml). Curve *C*: peak effluent fraction before incubation (peak *A* in Fig. 2) (μl/ml). Curve *D*: peak effluent fraction after incubation under same conditions as above (peak *B* in Fig. 2) (μl/ml). Curve *E*: purified urea-extracted pPTH (ng/ml).

Radioactivity in column effluent fractions was measured with a Nuclear-Chicago automatic gamma scintillation spectrometer. Protein was determined by the biuret method (16), with bovine serum albumin as a standard. The calcium concentration in saline extracts of parathyroid glands was measured by atomic absorption flame spectrophotometry (Perkin Elmer 303) after digestion of the extracts in boiling 14 N HNO_3–12 N $HClO_4$.

Biologic activity of PTH was studied by the method of Arnaud and coworkers (17) in 140-g thyroparathyroidectomized rats, which were kept conscious and perfused via the jugular vein and had been fed a low-calcium diet (General Biochemicals) for 4 days. Serum and urinary calcium were measured by atomic absorption spectrophotometry, and phosphorus was measured by an automated version of the Fiske and Subbarow (18) technique.

RESULTS

The standard radioimmunoassay curve produced by PTH in the 12,500 × *g* supernatant fraction of a saline extract of porcine parathyroid glands is shown as curve *A* in Fig. 1. After incubation of extracts for 6 hr at 37° in the presence of 5 mM EDTA, the immunologic characteristics of the extracts were changed (curve *B*) and closely resembled those of multiple dilutions of either hyperparathyroid serum or the PTH released into the medium by cultured parathyroid adenomata or porcine gland explants (3, 4, 6). The immunodilutional curves produced by both extracts differed from the curve produced by pPTH obtained from urea extracts of porcine parathyroid glands (curve *E*). When unincubated extracts were chromatographed on Sephadex G-50, the major peak of PTH immunoreactivity was eluted at a volume whose ratio to the void volume (v/v_0) was 1.21. This fraction produced an immunodilutional curve (curve *C*) that was almost identical to that of urea-extracted pPTH (curve *E*). However, after incubation, the major peak of immunoreactivity in the extracts eluted at a v/v_0 of 1.48 and produced curve *D*.

Gel filtration of extracts at 4° immediately after the addition of ^{131}I-bPTH as tracer substrate resulted in the elution pattern shown in Fig. 2 (*top*). More than 95% of the radioactivity added to the extracts was recovered in column effluents. The major peak of radioactivity (peak *A*) eluted at v/v_0 of 1.21. Incubation of ^{131}I-bPTH for up to 6 hr at 37° in buffer without the addition of parathyroid extract or with the addition of boiled extract failed to alter the elution pattern. However, when ^{131}I-bPTH was incubated at 37° with a saline parathyroid extract in the presence of EDTA or EGTA

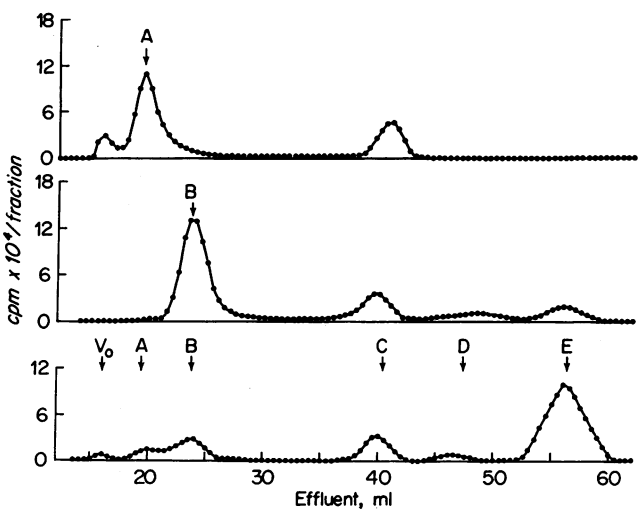

FIG. 2. Elution patterns [Sephadex G-50; 0.2 M ammonium acetate buffer (pH 4.7)] of saline parathyroid extracts containing ^{131}I-bPTH. *Top*, No incubation. *Middle*, 6-hr incubation, 5 mM EDTA (pH 6.0) at 37°. *Bottom*, 6-hr incubation, 1 mM $CaCl_2$ (pH 6.0) at 37°.

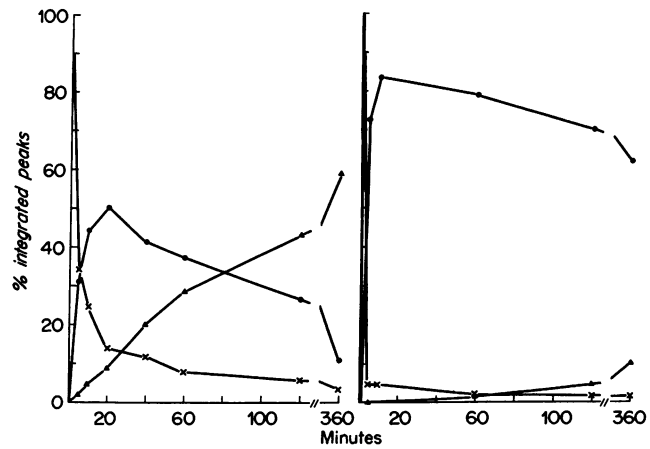

FIG. 3. Conversion of ^{131}I-bPTH by parathyroid extracts (incubated at pH 6.0, 37°) as a function of time. Peak fractions were separated by gel filtration on Sephadex G-50 in 0.2 M ammonium acetate buffer (pH 4.7). Total radioactivity in clearly identifiable peaks was determined by integration, and percentage of total radioactivity appearing in peak *A*(x), peak *B*(●), and peaks *D* + *E* (▲) was calculated. The remaining radioactivity was primarily in the void volume and ^{131}I peak. *Left*, With added calcium (1 mM). *Right*, With added EDTA (5 mM).

(5 mM) for 6 hr, the major peak of radioactivity shifted to a v/v_0 of 1.48 (peak *B*) [Fig. 2 (*middle*)]; two minor peaks (*D* and *E*) also became apparent. If EDTA was omitted and 1 mM calcium was added to the incubation mixture, *E* became the prominent peak of radioactivity [Fig. 2 (*bottom*)].

On the study of the various components by hydrodynamic flow chromatoelectrophoresis, peaks *A* and *B* remained at the origin, peak *C* migrated as if it were free iodide, and peak *E* migrated as "damaged" ^{131}I-bPTH (12). Peak *D* was not identified. The quantity of radioactivity in each peak was assessed by integration, and the percentage of the radioactivity present in each peak was calculated based on the radioactivity recovered in peak fractions (90%). Fractions containing the maximal radioactivity in each peak were incubated with excess concentrations of two anti-PTH antisera (GP 1M and Ch 14M). Only peaks *A* and *B* bound to either antibody; peaks *C*, *D*, and *E* were not immunoreactive (Table 1).

In the presence of 5 mM EDTA, the conversion of ^{131}I-bPTH to the smaller component by saline parathyroid extract was rapid, reaching a maximum (84%) at 10 min [Fig. 3 (*right*)]. The quantity of radioactivity in the nonimmunoreactive peaks (peaks *D* and *E*) was negligible, reaching only 10% of the total radioactivity after 6 hr of incubation. In contrast, incubation with 1 mM calcium resulted in a much slower conversion to the smaller species of PTH, reaching a maximum of 50% after 20 min, and a rapid appearance of nonimmunoreactive components (peaks *D* and *E*), which reached 60% after 6 hr [Fig. 3 (*left*)]. The amount of free ^{131}I (peak *C*) did not increase with time under either condition, indicating the absence of deiodinating activity in these extracts.

The effect of calcium on the peptidase activity of saline parathyroid extracts after 5 min of incubation is shown in Fig. 4. Peaks *D* and *E* were not formed during these short-term experiments. The conversion from peak *A* to peak *B* was decreased by the addition of calcium to 1 mM. In the presence of EDTA, conversion was virtually complete. Without EDTA or added calcium, there was some inhibition of the conversion reaction, presumably due to endogenous calcium (0.03 mM) in the saline parathyroid extract. The rate of conversion was not inhibited by the addition of either magnesium or barium to 1 mM.

The effects of temperature, extract concentration, and pH on the peptidase activity were studied in 5-min incubations in the presence of 5 mM EDTA and at pH 6.0 (except for pH studies). A decrease of the temperature to 20 and 5° decreased the rate of conversion by 50 and 70%, respectively.

TABLE 1. *% Binding of column peaks to excess anti-PTH antibody*

Peak	Antisera GP 1M (1:5000)	CH 14M (1:100)
A	84.3	75.0
B	88.2	78.7
C	0	2.9
D	...	1.0
E	4.8	2.0
^{131}I-bPTH	84.4	75.0

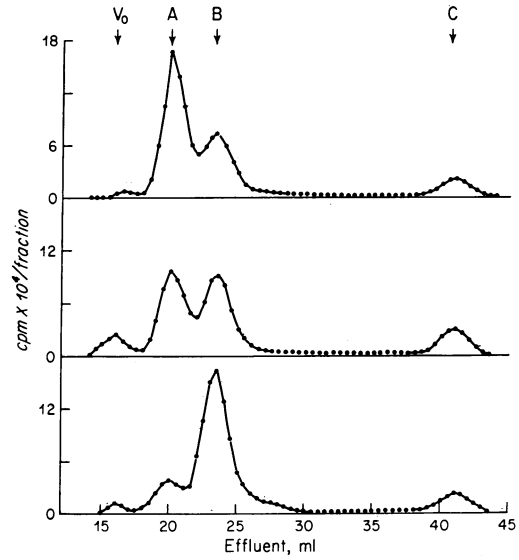

FIG. 4. Elution patterns [Sephadex G-50, 0.2 M ammonium acetate buffer (pH 4.7)] of saline parathyroid extracts containing ^{131}I-bPTH after 5 min of incubation (pH 6.0, 37°). *Top*, With 1 mM $CaCl_2$ added to incubation mixture. *Middle*, No addition (Ca = 0.03 mM). *Bottom*, With 1 mM EDTA added to incubation mixture.

At 50° the rate of conversion was decreased by 10%. The rate of conversion increased with larger quantities of extract added to the incubation mixture. Maximal peptidase activity occurred at pH values less than 6.0. No activity was observed at pH values above 7.0. Degradation into nonimmunoreactive components was minimal at all pH values to pH 8.0; at pH 9.0, it increased to 18%.

PTH peptidase activity in 10% saline extracts of 14 other porcine tissues was assayed by incubation with ^{131}I-bPTH for 5 min at pH 6.0 in the presence of EDTA, followed by gel filtration of the mixtures (Table 2). Conversion of ^{131}I-bPTH to a smaller species without significant production of nonimmunoreactive components was observed with extracts of liver, spleen, adrenal cortex, and pancreas. The peptidase activity in the liver extract showed similar sensitivity to added calcium as did that in the parathyroid. The influence of calcium on the activities of the other tissue extracts was not examined. The peptidase activities in these tissues were influenced differently by inhibitory agents. Trypsin inhibitor inhibited the conversion of ^{131}I-bPTH only in extracts of pancreas. Iodoacetate inhibited peptidase activity markedly in extracts of parathyroid, liver, and adrenal cortex, and less so in extracts of spleen and pancreas. Extracts of the other tissues studied produced little if any conversion in 5-min incubations but showed variable small amounts of conversion in longer incubations. Neither serum from a thyroparathyroidectomized pig nor the medium from cultures of human parathyroid tumor explants converted ^{131}I-bPTH during 60 min of incubation.

To test the biologic activity of the smaller-molecular-weight PTH component produced by PTH peptidase, 500 μg of bPTH, along with tracer amounts of ^{131}I-bPTH, was incubated with parathyroid extracts for 2 hr at 37° in the presence of 5 mM EDTA at pH 6.0. When the two components were separated by gel filtration, conversion was essentially complete, 85% of the radioactivity appearing in peak *B* and

TABLE 2. *Conversion of ^{131}I-bPTH by tissue extracts, serum, and parathyroid tissue culture medium, and effects of trypsin inhibitor and iodoacetate*

	Conversion, %*		
	No addition	Trypsin inhibitor (1 mg/ml)	Iodoacetate (1 mM)
Parathyroid	77	77	10
Spleen	52	52	31
Adrenal cortex	52	52	10
Liver	50	50	15
Pancreas	50	22	40
Tissues†	<10	—	—
Tissues‡	0	—	—
Porcine serum, thyroparathyroidectomized animal	0	—	—
Human parathyroid tissue culture medium	0	—	—

* Incubation for 5 min at 37° (pH 6.0), 5 mM EDTA; calculated as % of total radioactivity in smaller-molecular-weight form of PTH (peak *B*).

† Pituitary, thyroid, testis, thymus, and lung.

‡ Cardiac and skeletal muscle, kidney, adipose tissue, and brain.

less than 5% remaining in peak *A*. Fractions from the two radioactive peak areas were pooled separately. Radioimmunoassay of the two fractions showed the immunoreactivity in the peak-*B* fraction to be 1000 times that in the peak-*A* fraction and to have the immunologic characteristics of the converted molecule (curve *D*, Fig. 1).

An aliquot of the peak-*B* fraction, estimated on the basis of immunoreactivity and radioactivity in fractions to contain the converted equivalent of 20 μg bPTH, was perfused intravenously into thyroparathyroidectomized rats over a period of 4 hr (5 μg/hr). The characteristic effects of PTH were observed (Fig. 5): plasma calcium concentration increased significantly, there was a rapid increase in urinary phosphate, and urinary excretion of calcium decreased initially and then increased to twice the control value. In contrast, perfusion of the same volume of the peak-*A* fraction resulted in only minimal phosphaturia and no alterations in serum or urinary calcium. When 500 μg of bPTH was added to saline parathyroid extract and gel filtered on the same column without incubation, the perfusion of the equivalent of 5 μg/hr (total 20 μg) of bPTH from peak *A*, which contained 81% of the radioactivity and all of the immunoreactivity, produced biologic responses comparable to those shown in Fig. 5. Quantitation of the potency of PTH in this assay is not possible; it is used as a sensitive qualitative test to determine if both the renal and osseous effects of PTH are present in a given preparation. Both were present in the species of PTH formed by the PTH peptidase.

DISCUSSION

The PTH measured by radioimmunoassay in peripheral hyperparathyroid serum by some antisera has immunologic characteristics different from those of the human hormone previously extracted from parathyroid adenomata (molecular weight, 9500) (1–4). The PTH released by parathyroid adenoma explants in culture also has immunologic characteristics different from those of PTH extracted from glands but is indistinguishable from PTH found in serum (3, 4). Gel filtration of serum and parathyroid culture media have shown that, although both contain small amounts of the PTH molecule of molecular weight 9500, the predominant immunoreactive components in these materials are smaller molecules; in both cases they appear to have molecular weights in the range of 5000–7500 (2, 4, 5).†

The present report describes an enzymatic (peptidase) activity present in several tissues that rapidly converts PTH to a smaller-molecular-weight component, that has immunologic characteristics similar to those of PTH in plasma and in the medium of cultured parathyroid tissue. The activity is destroyed by boiling and is strongly inhibited by the sulfhydryl-reactive reagent, iodoacetate, but not by trypsin inhibitor. The species of PTH produced by this reaction has biologic activity, suggesting that it is a peptide fragment that contains the N-terminal region of the molecule (19). The PTH peptidase activity was highest in extracts of parathyroid gland but also was present in liver, adrenal cortex, and spleen. The properties of the enzymatic activity were studied primarily in extracts of parathyroid tissue. However, the activity found in liver extracts appeared to be identical in terms of its sensitivity to calcium and its susceptibility to inhibition by iodoacetate. Recent studies on serum obtained from parathyroid venous effluent suggest that when the PTH secreted from the parathyroid is rapidly removed from the tissue surface by blood flow, the conversion does not occur significantly until the molecule reaches the general circulation (2). Our studies show that serum itself does not produce the conversion. In view of the effective hepatic extraction of other polypeptide hormones (20, 21) and the high PTH peptidase activity found in liver extracts, it is likely that *in vivo* the conversion of PTH to the smaller species occurs predominantly in the liver.

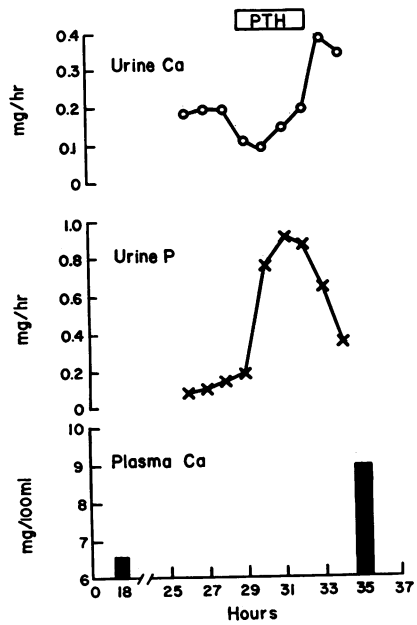

FIG. 5. Plasma calcium and urinary excretion of calcium and phosphorus in thyroparathyroidectomized rat perfused intravenously with the equivalent of 20 μg of converted bPTH (peak *B* in Fig. 2) over 4 hr.

The influence of calcium on the enzymatic cleavage of PTH by tissue extracts suggest that the serum calcium concentration may influence the metabolism of PTH, as well as the rate of its secretion. A low calcium concentration stimulates the formation of biologically active species, whereas a high calcium concentration accelerates the formation of smaller nonimmunoreactive, and presumably nonbiologically active, fragments. The increased formation of nonimmunoreactive PTH fragments during incubation with tissue extracts in the presence of calcium possibly reflects the activation by calcium of other proteases in the enzyme preparations. In contrast, the formation of a single, smaller-molecular-weight, immunoreactive component during extensive incubation in the presence of EDTA indicates that the peptidase activity represents a cleavage reaction of high specificity.

The presence of circulating fragments of polypeptide hormones has been considered to be a reflection of degradative processes that function primarily to dispose of the hormones. Our studies suggest that it may be important, in the case of PTH, to consider the possibility that a circulating, biologically active fragment of PTH may serve an important and unique physiologic function. The presence, in blood, of biologically active PTH species that have markedly different half-lives could add to the control of calcium and phosphate metabolism a dimension that could not be achieved by a single species with a short half-life. The two species in concert could provide both the constant background hormone concentration required to maintain a steady-state, normal serum calcium concentration and yet retain the potential of a rapid response to an acute hypocalcemic stimulus without resulting in a prolonged hypercalcemic overshoot.

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