Parathyroid Hormone Receptors of Renal Cortex: Specific Binding of Biologically Active, ^{125}I -Labeled Hormone and Relationship to Adenylate Cyclase Activation

[calcium metabolism/cyclic AMP/electrolytic iodination of peptides/
synthetic bovine parathyroid hormone(1-34)]

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ABSTRACT Biologically active ^{125}I -labeled bovine parathyroid hormone (prepared by electrolytic iodination) and its synthetic NH_2 -terminal (1-34) biologically active fragment bound rapidly and specifically to a purified plasma membrane preparation from bovine renal cortex. Binding of labeled intact hormone or labeled NH_2 -terminal (1-34) peptide was inhibited competitively by unlabeled (1-34) peptide in the same range of concentrations that activated renal cortical 3':5'-adenylate cyclase (EC 4.6.1.1) in these membranes. The concentrations of synthetic (1-34) peptide for half-maximal inhibition of binding of labeled hormone as well as half-maximal activation of the enzyme were about $0.6\ \mu\text{M}$ ($2.5\ \mu\text{g/ml}$). Therefore it is likely that the binding activity studied represents a physiologically important renal receptor for parathyroid hormone.

Biologically inactive (oxidized) forms of parathyroid hormone and (1-34) NH_2 -terminal peptide as well as calcitonin, glucagon, insulin, and epinephrine failed to competitively inhibit the binding of labeled (1-34) parathyroid hormone or activate adenylate cyclase in the renal cortical membrane preparation.

Observations with the NH_2 -terminal (1-34) biologically active fragment of parathyroid hormone suggest that the COOH -terminal region of the molecule is not required for receptor binding.

Parathyroid hormone (PTH) acts on the kidney to promote P_i excretion by inhibiting proximal tubular resorption of this ion (1, 2). The hormone has been thought to exert its effect through the intracellular mediator, adenosine 3':5'-cyclic monophosphate (cAMP) (3-9). In analogy with other cAMP-mediated hormonal systems (10), the sequence of events in PTH action on the kidney is generally presumed to involve (1) binding of the hormone to specific receptor sites on the renal cortical plasma membrane, (2) stimulation of cAMP formation by receptor-associated adenylate cyclase, and (3) cAMP action, on as yet unidentified intracellular systems, that leads ultimately to the known physiologic response.

Previous studies, both *in vivo* (3, 4) and *in vitro* (5-9), have established that PTH stimulates adenylate cyclase (EC 4.6.1.1) and increases cAMP in kidney. However, perhaps because of the difficulty of preparing biologically active isotopically labeled PTH (11), these studies have not characterized the first step in the above sequence: binding of the

hormone to specific receptor sites.* Conventional methods of labeling polypeptide hormones, such as the chloramine T procedure (12), require moderately strong oxidizing conditions, to which PTH is especially sensitive since oxidation of the methionine residue at position 8 destroys its biologic activity (13, 14). Moreover, it has been reported (15) that PTH labeled by the chloramine T procedure is immunologically altered.

The ^{125}I -labeled PTH and its NH_2 -terminal (1-34) peptide (bPTH 1-34) (16, 17) we used were prepared by the electrolytic iodination procedure of Sammon *et al.* (18) and had full biologic activity. The results of our studies† of the binding of these biologically active, labeled PTH preparations to a purified fraction of bovine renal cortical plasma membrane strongly suggest a close coupling between binding of hormone to its receptors and adenylate cyclase activation.

MATERIALS AND METHODS

PTH and Other Hormones. Bovine PTH was isolated and purified to homogeneity by the procedure of Brewer and Ronan (20). Homogeneous, biologically active, synthetic bPTH(1-34) (lot no. 31033) was a gift of Beckman Bioproducts (Palo Alto, Calif.). Homogeneous porcine calcitonin was prepared by the method of Arnaud and Tsao (21). Salmon calcitonin was a gift of Sandoz Pharmaceuticals (Hanover, N.J.). Bovine insulin (crystalline, lot no. 615-D63-5) and porcine glucagon (crystalline, lot no. 258-D30-138-4) were generously donated by Dr. R. W. Bromer of Eli Lilly Laboratories (Indianapolis, Ind.). Hydrogen peroxide-oxidized bPTH(1-34) and bPTH(1-84) were prepared by the method of Tashjian *et al.* (14). Epinephrine (*l*-isomer) was obtained from Sigma.

Renal Cortical Plasma Membranes. These were isolated from superficial layers (about 5 mm thick) of fresh bovine kidney cortex by the procedure of Fitzpatrick *et al.* (22) and were

Abbreviations: PTH, parathyroid hormone; bPTH(1-84), bovine parathyroid hormone, polypeptide chain of 84 amino acids; bPTH(1-34), amino-terminal tetratriacontapeptide of bovine parathyroid hormone (biologically active).

* After this manuscript was submitted for publication, a paper by Sutcliffe *et al.* appeared [*Biochem. J.* (1973) **134**, 913-921] in which the binding of biologically active ^{125}I -labeled bPTH (labeled by an enzymic method) to bovine renal cortical plasma membranes was described. Their findings are substantially in agreement with ours.

† These studies have been reported in Abstract form (19).

FIG. 1. Dose-response curves for bPTH(1-34) (●—●) and [125 I]bPTH(1-34) (○—○) prepared by electrolytic iodination in the bovine renal cortical plasma membrane adenylate cyclase assay. For [125 I]bPTH(1-34), the electrolytic iodination reaction mixture was assayed directly. Enzyme activity is expressed as percentage of the basal activity (10.9 ± 1.2 pmol of cAMP formed per min/mg of membrane protein). Each point is the mean $\pm$ SE for six determinations in two separate experiments.

stored in small portions at -85° until needed. Under such storage conditions, both PTH-binding activity and PTH-stimulated adenylate cyclase activity were stable for at least 6 months.

Analysis of our purified plasma membrane fraction revealed a 4.5-fold increase in the specific activities (for maximal response) for both PTH- and F $^+$ -stimulated adenylate cyclase, compared to the crude homogenate of renal cortex. The specific activity of ouabain-sensitive ATPase [a marker enzyme for antiluminal plasma membranes (8)] increased 7-fold. Other marker enzyme studies confirmed that our preparation of plasma membranes was substantially free of contamination from other subcellular fractions (22).

Adenylate Cyclase Assay. In principle, the method of Bär and Hechter (23) was used with [α - 32 P]ATP as substrate. The composition of the enzyme incubation mixture was as described (24). The labeled cAMP formed in the assays was separated from other radioactive compounds by thin-layer chromatography on 10×20 -cm sheets of polyethyleneimine with 0.25 M LiCl as developing solvent (25). Radioactivity in cAMP and ATP areas of the chromatogram was measured in a scintillation counter (Nuclear-Chicago). Enzyme activity was expressed as pmol of cAMP produced per min/mg of protein.

Preparation of Labeled PTH. The electrolytic iodination procedure was used as described by Sammon *et al.* (18). In a typical labeling experiment, 90 μ g of PTH (0.01 μ mol) was added to 4–5 mCi of 125 I $^-$ (carrier-free, Union Carbide) and 0.01 μ mol of KI in 4 ml of 50 mM potassium phosphate buffer

FIG. 2. Time course of binding of [125 I]bPTH(1-84) to bovine renal cortical plasma membranes. Membrane protein concentration was 190 μ g/ml (15.2 μ g total) and [125 I]bPTH(1-84) concentration was about 9 nM. Each point is the mean $\pm$ SE for triplicate determinations in a single experiment.

(pH 7.6) in a platinum crucible. A current (40 μ A) was passed through the stirred solution at 25° for 25 min. The labeled PTH was freed of "damaged hormone" and unreacted 125 I $^-$ by cellulose chromatography and elution with human serum as described by Berson and Yalow (26). The labeled hormone (estimated specific activity, 10–20 μ Ci/ μ g) was stored in small portions at -20° and used within 3 weeks; once thawed, it was never refrozen for subsequent experiments. The (1-34) peptide was similarly labeled and stored (estimated specific activity, 2–10 μ Ci/ μ g).

Binding Assays. The method of Rodbell *et al.* (27) was used with minor modifications. Immediately after thawing, the renal plasma membranes were diluted with water to a protein concentration of 760 μ g/ml. Membranes (20 μ l, 15.2 μ g of protein) were added to soft-glass incubation tubes containing 40 μ l of 0.1 M Tris buffer (pH 7.5) with 12.5% bovine-serum albumin (Sigma) to decrease nonspecific adsorption of hormone to the glass surfaces. Unlabeled PTH (10 μ l), when needed, was then added and the tubes were incubated at 0° for 5 min. Labeled PTH (10 μ l, about 2×10^5 cpm) was added, giving a final labeled PTH concentration of 6–12 nM. All determinations were carried out in triplicate. After 15 min of incubation at 30° , 50- μ l aliquots were layered onto 0.3 ml of 20 mM Tris buffer (pH 7.5) containing 2.5% albumin in plastic "microfuge" tubes. The tubes were centrifuged (Beckman model 152; 15,000 rpm) for 5 min at 4° and the supernates were removed by gentle aspiration. The surfaces of the tiny pellets were rinsed with ice-cold 10% sucrose and the tubes were recentrifuged. After a second aspiration of the supernates, the tips of the tubes (containing the pelleted membranes) were cut off and counted in a well-type gamma counter.

In expressing the data, radioactivity values (about 10% of total counts in the pellet) for blank determinations (containing no membranes) were subtracted from each experimental determination to yield a value for net radioactivity bound to membranes.

Miscellaneous. Protein was determined by the method of Lowry *et al.* (28) with bovine-serum albumin as standard. Sodium dodecyl sulfate (final concentration, 0.2%) was added to solubilize membrane proteins.

RESULTS

Biologic Activity of Iodinated PTH and Synthetic (1-34) Active Peptide. Both bPTH(1-84) and bPTH(1-34) were subjected to the electrolytic iodination procedure but with 127 I $^-$ instead of 125 I $^-$. In the renal cortical membrane adenylate cyclase assay, the dose-response curves for the iodinated and for the uniodinated bPTH(1-34) were similar but not identical (Fig. 1). Such also was the case for electrolytically iodinated bPTH(1-84): the concentrations of both the iodinated and uniodinated untreated species required for half-maximal activation of the renal cortical adenylate cyclase were both 320 nM (3.0 μ g/ml). When [125 I]bPTH(1-84) was assayed directly, it had virtually the same ability to activate adenylate cyclase as did unlabeled hormone at equal concentrations. Thus, any alterations in the activities of bPTH(1-84) or bPTH(1-34) that might have been produced by the electrolytic iodination procedure are minor, and the hormone retains essentially full biologic activity in the renal

cortical adenylate cyclase assay system when labeled by this method.

Binding and Adenylate Cyclase Activation. With 15.2 μ g of plasma membrane fraction, about 6% of the [125 I]bPTH(1-84) added was rapidly bound. Maximal binding with this amount of membranes occurred at 10–15 min (Fig. 2); the binding of [125 I]bPTH(1-34) showed a similar time course. The proportion of 125 I-labeled bPTH(1-84) binding to the renal cortical plasma membrane fraction showed a nearly linear dependence on membrane concentration; 122 μ g of membrane protein bound about 40% of the added [125 I]bPTH(1-84). It was necessary to perform the binding experiments with 15.2 μ g of membrane to minimize effects of non-specific binding of labeled PTH and trapping of isotope in the membrane pellets. Furthermore, by use of this low amount, the conditions of the binding assays were such that there was direct competition between labeled and unlabeled species for a limited number of receptor sites.

The binding of [125 I]bPTH(1-34) or [125 I]bPTH(1-84) was competitively inhibited, in dose-dependent fashion, by increasing concentrations of unlabeled bPTH(1-34) (Fig. 3). For half-maximal inhibition of binding of labeled peptide to membranes, the concentration of unlabeled bPTH(1-34) was about 750 nM for [125 I]bPTH(1-34) and about 500 nM for [125 I]bPTH(1-84).

The dose-response curve for cAMP production stimulated by bPTH(1-34) demonstrates a close correlation between the inhibition of binding of labeled hormone and the extent of adenylate cyclase activation in this receptor system (Fig. 4). The concentrations of unlabeled bPTH(1-34) required for half-maximal adenylate cyclase activation were about 600 nM [2.5 μ g bPTH(1-34)/ml].

Specificity Studies. Insulin, glucagon, calcitonin (porcine or salmon), or epinephrine (all at concentrations from 10 to 70,000 nM) did not significantly inhibit the binding of [125 I]bPTH(1-34) or [125 I]bPTH(1-84) to the cortical plasma membrane fraction. The biologically inert analogs, hydrogen peroxide-oxidized bPTH(1-34) and bPTH(1-84), were similarly without effect. None of these hormones significantly stimulated cAMP formation in renal cortical plasma membranes at concentrations of 10–7000 nM.

DISCUSSION

Using biologically active 125 I-labeled parathyroid hormone (PTH), we have demonstrated specific binding of the hormone to a plasma membrane fraction isolated from one of its target tissues, renal cortex. Because the dose-response characteristics of binding closely correlated with the activation of adenylate cyclase in the same membranes, it is likely that the binding component in the membrane fraction represents a physiologically important receptor of PTH.

A prime requirement for the direct study of hormone-receptor interactions *in vitro* is a labeled hormone that has high specific radioactivity, yet retains full biologic activity. The electrolytic iodination procedure developed by Sammon *et al.* (18) was shown by them to produce a labeled preparation of PTH that had full biologic activity when assayed in three different systems (liver mitochondria, embryonic rat bone in tissue culture, and the calcium mobilization assay in thyroparathyroidectomized rats). Our results with a fourth system, the renal cortical membrane adenylate cyclase assay,

FIG. 3. Inhibition of binding of [125 I]bPTH(1-34) (A) and [125 I]bPTH(1-84) (B) to renal cortical plasma membranes by increasing concentrations of unlabeled synthetic bPTH(1-34). Each point represents the mean $\pm$ SE of six determinations in two separate experiments. The data are expressed as the percentage of maximal binding of labeled hormone (100% represents 6% of the total 125 I counts added).

support their findings with bPTH(1-84) and also indicate that it is possible to prepare biologically active synthetic [125 I]bPTH(1-34) by the same procedure.

In our experiments, we found that unlabeled synthetic bPTH(1-34) inhibited the binding, to renal cortical membranes, of [125 I]bPTH(1-84) and [125 I]bPTH(1-34) equally well. However, in preliminary experiments not reported here, we found that unlabeled bPTH(1-84) was far less effective on an equimolar basis than unlabeled bPTH(1-34) in inhibiting the binding of labeled bPTH(1-34). There are several possible explanations for this phenomenon. The natural bPTH(1-84) that we used might have undergone partial denaturation during its isolation and therefore was less able to bind to receptors. This does not appear likely because the [125 I]bPTH(1-84) consistently bound to membranes and bPTH(1-34) inhibited its binding to the same extent as it did the binding of [125 I]bPTH(1-34).

An attractive alternative explanation is that the renal cortical membrane PTH receptor is capable of binding only a limited region of the PTH(1-84) sequence (presumably the

FIG. 4. Comparison of ability of unlabeled synthetic bPTH(1-34) to bind to bovine renal cortical plasma membranes ($\bullet$ — $\bullet$) and ability to activate adenylate cyclase ($\circ$ — $\circ$). Binding data are plotted as percentage of maximal binding in absence of unlabeled bPTH(1-34) (about 6% of total radioactivity bound under assay conditions); each point is the mean $\pm$ SE of six determinations in two separate experiments. Adenylate cyclase activity is expressed as percentage of maximal response (basal activity, 10.9 ± 1.2 pmole of cAMP formed per min/mg of membrane protein); each point is the mean $\pm$ SE for six determinations in two separate experiments.

NH₂-terminal region). Such a hypothesis would require the existence of a mechanism for the cleavage of PTH(1-84) concomitant with or subsequent to its binding. In fact, in preliminary experiments we have observed that [¹²⁵I]bPTH(1-84) is partially cleaved when incubated with membranes and that significant inhibition of binding of [¹²⁵I]bPTH(1-34) by bPTH(1-84) could only be achieved by preincubating the unlabeled bPTH(1-84) with membranes whereas bPTH(1-34) inhibited binding without such preincubation. These considerations do not exclude the possibility that the hypothetical "receptor-active" fragment of the PTH molecule could arise at a site(s) remote from the renal receptor.

Our results indicate that specific receptors for PTH and its NH₂-terminal "active peptide" exist on bovine renal cortical plasma membranes. These receptors bind the hormone rapidly and are closely coupled to a PTH-sensitive adenylate cyclase in these membranes. In future studies it will be important to identify the means by which the binding of hormone to its renal receptors is regulated and how such regulation relates to activation of the adenylate cyclase in these membranes. This information could contribute significantly to our understanding, at the molecular level, of certain disorders of parathyroid hormone, calcium, and phosphate metabolism.

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