Analysis of the Requirements for Parathyroid Hormone Action in Renal Membranes with the Use of Inhibiting Analogues*

(Received for publication, September 12, 1974)

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Two synthetic analogues of bovine parathyroid hormone (PTH) with NH₂-terminal modifications, PTH-(3-34) and [desamino-Ala-1]PTH-(1-34), were found to lack agonist activity but to demonstrate antagonist properties when tested in the rat renal cortical adenylyl cyclase assay in vitro against the native hormone or against PTH-(1-34), the active synthetic NH₂-terminal tetradecapeptide. The inhibition exhibited by these analogues was proportional in degree to the dose of inhibitor, abolished by oxidation of the analogue, reversible by addition of an excess of active hormone, and specific for parathyroid hormone-stimulated renal adenylyl cyclase. No inhibition of basal or sodium fluoride-stimulated renal adenylyl cyclase could be demonstrated. Two other synthetic bovine analogues, PTH-(13-34) and PTH-(1-26), were devoid of agonist and antagonist properties.

The over-all results suggest that the requirements for receptor binding of parathyroid hormone are rather broad. Conformational factors or binding interactions involving specific residues, or both, seem to require the entire sequence from residue 3 to residue 27 for receptor binding to occur. A dichotomy between receptor binding and adenylyl cyclase activation was demonstrated only by alterations or deletions involving the first 2 NH₂-terminal residues of the hormone and emphasizes the importance of these residues in eliciting the biological activity of parathyroid hormone. The two antagonists, [desamino-Ala-1]PTH-(1-34) and PTH-(3-34), should be useful in further analysis of the initial steps in hormone action.

Bovine (1-3), porcine (4, 5), and human (6) parathyroid hormones have been established to be single chain polypeptides consisting of 84 amino acids. The structural requirements necessary for virtually full biological activity (in each species) (7-10) are present in residues 1 through 34 in the NH₂-terminal region of the molecules, and the structure (in the bovine species) necessary for a minimum of biological activity is apparently in a continuous sequence extending from positions 2 through 27 (10).

The early events in the mechanism of action of parathyroid hormone are currently believed to involve binding to specific receptor sites in cell membranes of target tissues (11-13), with subsequent stimulation of adenylyl cyclase in these membranes (14-16). Stimulation of this enzyme in cell homogenates (17, 18) or membrane preparations (16) has been employed as a biosay in vitro for the hormone.

The studies reported here were performed using synthetic peptide fragments of bovine parathyroid hormone of markedly reduced or no biological activity to further elucidate relationships of structure to activity by measuring, as a biological response, the activation of rat renal cortical adenylyl cyclase in vitro. Two bovine fragments, [desamino-Ala-1]PTH-(1-34) and PTH-(3-34), were shown to inhibit parathyroid hormone-stimulated adenylyl cyclase. These studies have enabled analysis of the structural requirements for receptor binding and adenylyl cyclase activation.

EXPERIMENTAL PROCEDURE

Hormone Preparations—Synthesis of bovine parathyroid hormone fragments PTH-(1-34), PTH-(3-34), PTH-(1-26), PTH-(13-34), and [desamino-Ala-1]PTH-(1-34) was carried out by a modification (10, 19) of the Merrifield procedure for solid phase synthesis (20) using the Beckman model 990 peptide synthesizer, and fragments were purified as previously described (10, 19). All peptides were shown to correspond to theoretical composition by acid and enzymic hydrolysis techniques followed by automated amino acid analysis (1, 7), and, as well, were shown to be structurally homogeneous by complete sequence analysis employing repetitive Edman degradation (19).

Native bovine PTH (1-84) used as a standard in the adenylyl cyclase assay for potency determination was Medical Research Council (MRC) Research Standard, Lot No. MRC 72/286 (National Institute for Medical Research, London, England). For all other studies, purified bovine parathyroid hormone 1 (approximately 3000 MRC units/mg) (21) was prepared from the trichloroacetic acid precipitate of bovine parathyroid gland extracts (22) by gel filtration and ion exchange chromatography on carboxymethylcellulose as previously described (1).

Synthetic salmon calcitonin was the gift of Dr. J. Bastian of Armour.* This work was supported by National Institutes of Health Research Grants AM11794-06 and AM04501 and by the John A. Hartford Foundation Inc. and the National Aeronautics and Space Administration.

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The abbreviations used are: PTH, parathyroid hormone (bovine); cyclic AMP, cAMP, cyclic adenosine 3′-5′-monophosphate.
Pharmaceutical Co. Insulin A chain, carboxymethylated and reduced, was obtained from Schwarz/Mann Research Laboratory. Whale apomyoglobin was obtained from Beckman Instruments, Spinco Division, Palo Alto, Calif.

Oxidized bovine parathyroid hormone fragments were prepared according to a modification of a method previously described (17) by incubating 1 mg/ml of fragment for 1 hour in 0.1 N acetic acid and 1% H2O2 (w/w) at 25°C. Incubation was terminated by diluting the reaction mixture with distilled water and immediately freezing and lyophilizing this mixture. Treatment of the 1-84 parathyroid hormone molecule in this way resulted in complete loss of the hormone's capacity to stimulate renal adenyl cyclase.

**Renal Cortical Membrane Preparation**—Differential centrifugation and ultracentrifugation on continuous sucrose density gradients were employed to prepare highly purified renal cortical membranes from male Sprague-Dawley rats (150 to 200 g) according to the method of Marx et al. (16).

**Assay of Adenylyl Cyclase Activity**—The method used to assay adenyl cyclase activity was a modification of that previously described (17, 18, 23). Unless otherwise specified, the incubation mixture for measurement of adenyl cyclase activity contained, in a 0.17-ml volume, 50 mM Tris-HCl, pH 7.4, 0.84 mM ATP, 0.8 to 2.0 x 10^(-6) cpm of a labeled [32P]ATP (New England Nuclear), 9 mM theophylline, 4.2 mM MgCl2, 26 mM KCl, 0.115% albumin, an ATP-regenerating system consisting of 5 mM creatine phosphate (Schwarz/Mann), and 0.1 mg/ml of creatine phosphokinase, and 30 to 80 µg of membrane protein. Hormones, non-hormonal polypeptides, or sodium fluoride were added in 5-µl volumes and were added simultaneously when two of these were incubated together. Incubations were performed at 37°C and were initiated by addition of membranes. Incubations were terminated by addition of a 100-µl solution containing 12.5 mM Na2HPO4 (H+ form; 200 to 400 mesh, J. T. Baker) columns and recovered from the supernatant after two precipitations with ZnSO4 and Ba(OH)2 as previously described (17, 18, 23). Recoveries of [3H]cAMP were about 60%.

Protein was determined by the method of Lowry et al. using bovine serum albumin as a standard (34).

**RESULTS**

Fig. 1 illustrates the structures, and Table I lists the in vitro rat kidney adenyl cyclase potencies of the various bovine parathyroid hormone analogues used in these studies. All analogues other than PTH-(1-34) employed in these studies were of minimal potency or essentially inert in this in vitro assay.

**Antagonism of Parathyroid Hormone Action**—Fig. 2 depicts the activation of adenyl cyclase by increasing concentrations of PTH-(1-84) in the absence and in the presence of a 63.5 µM concentration of PTH-(3-34). Marked inhibition of the action of PTH-(1-84) by this analogue is evident. In contrast, two control proteins (whale apomyoglobin and a modified insulin A chain) produced either minimal or no inhibition of the action of PTH-(1-84). No inhibition was obtained with other nonparathyroid-related proteins tested, including a collagen fragment and bovine serum albumin. Contrasting with the apomyoglobin effect, the inhibition by PTH-(3-34) of the biological activity of the PTH-(1-84) molecule was found to be dose-related, and oxidation of PTH-(3-34) resulted in loss of this inhibitory property (Fig. 3).

The peptide fragment [desamino-Ala-1]PTH-(1-34) was found to similarly reduce PTH-(1-84)-stimulated adenyl cyclase activity (with loss of this effect after oxidation) in experiments of the same nature.

When a 300-fold excess of PTH-(3-34) was added, after 3½ min, to a native parathyroid hormone-stimulated adenyl cyclase assay, a marked drop in hormone-stimulated enzymatic activity was noted, demonstrating the rapid reversibility of the interaction between parathyroid hormone and receptor under these circumstances (Fig. 4). The further addition of excess PTH-(1-84) then resulted in further activation of the enzyme. The inhibition produced by the antagonist is therefore surmountable.

Both PTH-(3-34) and [desamino-Ala-1]PTH-(1-34) produced inhibition of adenyl cyclase activated by PTH-(1-34), the active moiety of PTH-(1-84) (Fig. 5); the inhibitory property was again abolished by prior oxidation of both inhibiting analogues.

**Site of Action and Specificity of Inhibitors**—When a concentration of PTH-(1-84) that activates adenyl cyclase submaximally in these membranes is added to a concentration of sodium fluoride (8 mM) found to maximally stimulate the enzyme, no additional adenyl cyclase activity results (Fig. 6),
FIG. 3. Effect on PTH-(1-84)-stimulated adenylyl cyclase activity of oxidized PTH-(3-34) and unoxidized PTH-(3-34). Adenylyl cyclase activity, stimulated by increasing concentrations of PTH-(1-84), was determined in the absence of PTH-(3-34) (○); in the presence of oxidized PTH-(3-34), 80 μM (●); and in the presence of unoxidized PTH-(3-34), 20 μM (■), 40 μM (▲), and 80 μM (▼). The method of assay of adenylyl cyclase activity is described in the text. Each point is the mean of triplicate determinations.

FIG. 4. Generation of cAMP with time before and after successive addition of PTH-(1-84) and PTH-(3-34). Renal cortical membranes (0.5 mg/ml) were incubated for adenylyl cyclase activity in the final incubation volume of 2.7 ml. PTH-(1-84) and PTH-(3-34) (inhibitor) were sequentially added, after removal of 100-μl aliquots, in volumes of 0.005 ml and 0.025 ml to give final concentrations of 0.2 μM and 63.5 nM, respectively. PTH-(1-84) was then added in 0.02 ml to give a final concentration of 10 μM. Production of cAMP was followed by removing, at 30-s intervals, 100-μl aliquots and determining [3P]cAMP formed from [α-32P]ATP as described under “Experimental Procedure.” The remainder of the experimental conditions are given under “Experimental Procedure.”

suggesting that sodium fluoride and PTH-(1-84) activate the same enzyme. Neither PTH-(3-34) nor [desamino-Ala-1]-PTH-(1-34) significantly inhibited fluoride-stimulated adenylyl cyclase activity (Fig. 6), and neither inhibits basal adenylyl cyclase activity; consequently, the inhibition seemed not to be of the adenylyl cyclase enzyme itself.

Neither fragment inhibited salmon calcitonin-stimulated adenylyl cyclase activity (Fig. 7), indicating that the inhibition was specific for parathyroid hormone-stimulated adenylyl cyclase (16).

Structural Requirements of Inhibitor Fragments—In Fig. 8, four synthetic parathyroid hormone fragments are compared with respect to their inhibitory properties. Increasing concentrations of fragments were added to a concentration of PTH-(1-34) that produces half-maximal stimulation of adenylyl cyclase in these membranes. Only PTH-(3-34) and [desamino-Ala-1]-PTH-(1-34) produced significant inhibition.

DISCUSSION

The specificity of the inhibition by the analogues PTH-(3-34) and [desamino-Ala-1]-PTH-(1-34) for PTH-stimulated adenylyl cyclase activity and the absence of inhibition of fluoride-stimulated and basal adenylyl cyclase activity are consistent with a receptor locus of action. The loss of inhibitor properties after oxidation of these peptides is analogous to the loss of stimulating activity evidenced by PTH agonists after oxidation, and in both cases is probably due to steric hindrance to receptor binding secondary to the introduction of bulky sulfoxide and sulfone groups at the methionine residues at positions 8 and 18 (25). In contrast to PTH-(3-34),
PTH-(3-34) does not stimulate adenylyl cyclase, but can inhibit the action of PTH agonists, this analogue retains sufficient features for receptor binding.

Consequently, a continuous sequence from residue 3 through residue 13 appears to be a true antagonist, [desamino-Ala-1]PTH-(1-34). Adenylyl cyclase activity stimulated by increasing concentrations of salmon calcitonin (CT), was determined in the absence of PTH analogues (Δ); in the presence of 60 μM PTH-(3-34) (♀); and in the presence of [desamino-Ala-1]PTH-(1-34) (O). The method of assay of adenylyl cyclase activity is described in the text. Each point is the mean of triplicate determinations.

which appears to be a true antagonist, [desamino-Ala-1]PTH-(1-34) has documented potency in vivo (26) and may therefore be a weak “partial agonist” (27, 28) in vitro in renal membranes, effectively competing at high molar ratios with PTH-(1-34) or PTH-(1-34) for receptor sites, but stimulating adenylyl cyclase only minimally.

Since PTH-(1-34) exhibits approximately 100% of the activity of the native hormone, it must contain virtually all of the requirements necessary for optimal receptor binding and adenylyl cyclase stimulation in these membranes. Since PTH-(3-34) does not stimulate adenylyl cyclase, but can inhibit the action of PTH agonists, this analogue retains features adequate for receptor binding but not those requisite for cyclase activation. The role of the NH₂-terminal 2 residues of the hormone in stimulating PTH-sensitive adenylyl cyclase is at present unknown. Since PTH-(3-34) does not inhibit PTH-(1-34) on an equimolar basis, deletion of the first 2 residues must lower avidity of receptor binding. A unique allosteric role for these residues in linking the receptor to the catalytic component of the cyclase system cannot be excluded, however. It has now been demonstrated for a number of peptide hormones that affinity for a target-tissue receptor is not alone sufficient for expression of a biological response (29-31). Alteration (as in [desamino-Ala-1]PTH-(1-34)) or complete deletion of the 1st residue seriously diminishes biological potency, but it is only when the 2nd residue, valine, is also deleted that complete loss of activity both in vivo and in vitro occurs (10), suggesting that residue 2, which is conserved in all species so far examined (2, 3, 6, 10), may be of at least equal importance with residue 1 in the interaction of the hormone with the cell membrane.

If the active moiety PTH-(1-34) is progressively shortened from the COOH terminus, fragments of decreasing potency are produced until the fragment PTH-(1-26) results, which neither stimulates nor inhibits in the adenylyl cyclase assay (10). Receptor binding apparently occurs when the COOH terminus is extended to include the sequence 27-34, as in PTH-(3-34). Therefore, the decreasing potency of analogues shortened from the COOH terminus appears to be due to decreasing receptor affinity. Only at the NH₂ terminus can a significant dichotomy between receptor binding and stimulation of adenylyl cyclase be demonstrated.

The structural requirements for receptor binding appear rather broad. Thus, in addition to residues 1 and 2 and residues within the 27-34 sequence, those within the 3-12 sequence may be of importance for binding, since progressive shortening of the NH₂ terminus to position 13 results in a fragment, PTH-(13-34), that does not inhibit and, therefore, does not bind with detectable affinity. Conformational requirements may also be significant in the membrane environment, as suggested by the observation that the combination PTH-(1-12) and PTH-(13-34) does not stimulate adenylyl cyclase (10), despite the presence of the entire sequence of residues required both for binding and activation. Since earlier studies demonstrated that PTH-(1-27) has weak agonist properties in the renal adenylyl cyclase assay in vitro (10), we can conclude that this analogue retains sufficient features for receptor binding.

Consequently, a continuous sequence from residue 3 through 27 appears to contain the minimum structural and conformational requirements for receptor binding.

The analogues of parathyroid hormone shown in these studies to inhibit PTH-stimulated adenylyl cyclase in renal cortical membranes may serve as models for the synthesis of additional inhibitory analogues. Such derivatives with antagonist properties can be used as probes to analyze the molecular nature of the interactions between parathyroid hormone and renal cell membranes and should provide further insight into the initial steps in hormone action.

REFERENCES

Additions and Corrections


Requirements for Parathyroid Hormone Action in Renal Membranes with the Use of Inhibiting Analogues.


On Page 3199, the name of the first author should be David Goltzman

Page 7979, Step 6, third sentence should read, instead of “. . . from the cathodal end. . .”: Aldolase was eluted from the anodal end . . .

Vol. 250 (1975) 3199-3203

In Goltzmann, David, André Peytreman, Edward Callahan, Geoffrey W. Tregear, and John T. Potts, Jr. Analysis of the

In Yamasaki, Nobuyuki, Juichiro Shimanaka, and Martin Sonenberg. Studies on the Common Active Site of Growth Hormone. Revision of the Amino Acid Sequence of an Active Fragment of Bovine Growth Hormone.

Page 2512, Table III, Line 2, arginine residues under Column 4, peptide C-1-4, should read 0 (0)

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Analysis of the requirements for parathyroid hormone action in renal membranes with the use of inhibiting analogues.
D Goltzmann, A Peytreman, E Callahan, G W Tregear and J T Potts, Jr