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Inhibition of the *in vivo* parathyroid hormone-mediated calcemic response in rats by a synthetic hormone antagonist

SAMUEL H. DOPPELT*†, ROBERT M. NEER*, SAMUEL R. NUSSBAUM*, PANFILO FEDERICO*, JOHN T. POTTS, JR.*, AND MICHAEL ROSENBLATT‡

*Endocrine and Mineral Metabolism Units, Massachusetts General Hospital, and Department of Medicine, Harvard Medical School, Boston, MA 02114; and ‡Merck Sharp & Dohme Research Laboratories, West Point, PA 19486

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ABSTRACT **The parathyroid hormone (PTH) analog, [Tyr^{34}]bovine PTH-(7–34)-amide, can inhibit the PTH-mediated elevation of plasma calcium in thyroparathyroidectomized rats *in vivo*. The analog is devoid of PTH-like agonist activity in this system. Repeated doses of analog inhibit the animal's calcemic response to PTH. The elevation in serum calcium levels mediated by PTH in this assay reflects PTH action (calcium mobilization) on bone. Earlier studies demonstrated antagonist properties of the analog in a renal-based assay; PTH-stimulated increases in urinary phosphate and cyclic AMP excretion were completely inhibited by the synthetic analog. Along with previous studies, this report indicates that [Tyr^{34}]bovine PTH-(7–34)-amide is an effective *in vivo* antagonist for several major parameters of PTH action in both kidney and bone.**

Parathyroid hormone (PTH) is a single-chain polypeptide of 84 amino acids that serves a critical role in calcium and phosphate homeostasis through its actions on kidney and bone (1–3). Extensive structure–activity studies of PTH based on chemical synthesis of multiple fragments and analogs of the hormone and evaluation of their properties in several PTH bioassay systems have delineated within the hormone molecule separable domains responsible for receptor binding and activation (4–6). Structural modification of the amino terminus of PTH by substitution or amino-terminal truncation generates hormone analogs that bind to, but do not activate, PTH-specific receptors and, therefore, act *in vitro* as competitive antagonists of PTH action (7, 8).

One such analog, [Tyr^{34}]bPTH-(7–34)-amide (where b stands for bovine), is the first PTH analog shown to possess antagonist properties *in vivo* (9) (Fig. 1). Because supplies of the synthetic peptide were limited and efforts were directed at demonstrating inhibitory efficacy of the analog (hence it was used in large molar excess compared to PTH), the assay system employed in these earlier studies was selected to assess the effects of the analog on a major, but rapid-response parameter of PTH action. PTH acts on the kidney to stimulate urinary excretion of phosphate and cyclic AMP within minutes of its intravenous administration. Using thyroparathyroidectomized rats, [Tyr^{34}]bPTH-(7–34)-amide was shown to inhibit completely PTH-stimulated increases in renal phosphate and cyclic-AMP excretion *in vivo* (9). In this assay system, however, determination of the effects of this analog on PTH-stimulated calcemic responses was precluded because the thyroparathyroidectomized rats required maintenance by an intravenous calcium administration.

The utility of such a synthetic antagonist of PTH for studies of the mechanism of action of the hormone and for potential clinical applications in disorders of calcium metabolism would be far greater if its antagonist properties included the

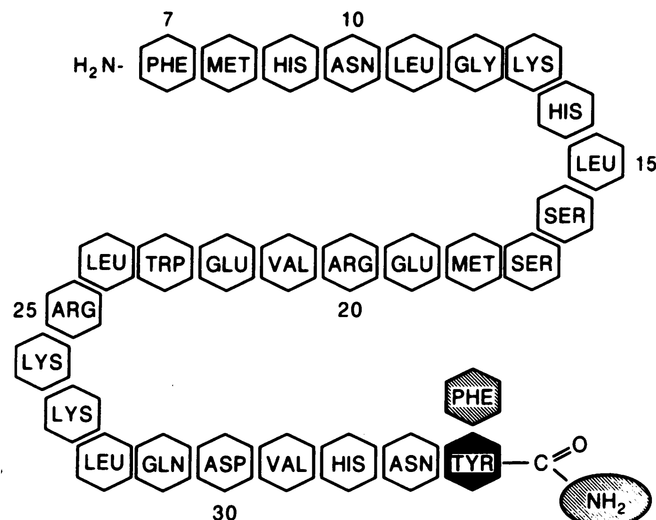

FIG. 1. Amino acid sequence of the parathyroid hormone antagonist effective *in vivo*, [Tyr^{34}]bPTH-(7–34)-amide. The cross-hatched regions depict substitutions for the native sequence: tyrosine is substituted for phenylalanine at position 34 and the carboxyl terminus is carboxylamide.

bone and calcemic effects of PTH. To test for the biological properties of this synthetic analog on the PTH-mediated calcemic response, a rapid and sensitive assay was designed (10). This assay minimizes the quantities of antagonist required for *in vivo* evaluation, is rapid, and is primarily bone dependent. Also, the assay is structured to give a potential antagonist an advantage over PTH during the course of the experiment: i.e., the assay is designed to identify PTH antagonists effective *in vivo*. The experiments described in this report evaluated the inhibitory effects of [Tyr^{34}]bPTH-(7–34)-amide in this test system.

METHODS

The PTH antagonist [Tyr^{34}]bPTH-(7–34)-amide and the synthetic fragment human (h) PTH-(1–34) were synthesized by the solid-phase method of Merrifield and co-workers (11–13) using modifications described (14). The peptides were purified by gel-filtration chromatography followed by semipreparative HPLC (15, 20). hPTH-(1–34) and the antagonist were dissolved in diluent immediately prior to use.

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Abbreviations: PTH, parathyroid hormone; hPTH, human PTH; bPTH, bovine PTH; hPTH-(1–34), hPTH fragment (residues 1–34); MRC, Medical Research Council (United Kingdom).

†To whom reprint requests should be addressed at: Mineral Metabolism Unit, Bulfinch 3, Massachusetts General Hospital, Boston, MA 02114.

The test system employed male rats 130–150 g (Charles River Breeding Laboratories) maintained on a calcium-free diet for 6.5 days and fasted the seventh night (10). On the following day the animals underwent thyroparathyroidectomy under ether anesthesia. One hour later two-thirds of the rats received 5 units of hPTH-(1–34) plus 68 μmol of calcium chloride by tail vein; one-third received only 68 μmol of calcium chloride (control). The injection volume was 0.6 ml, and the diluent for all injections contained heat-inactivated human serum albumin (1 mg/ml), ε-aminocaproic acid (5 mg/ml) (16), and NaCl (9 mg/ml). Under such circumstances the administration of diluent alone results in a significant decline in serum calcium concentration at 1 and 2 hr after treatment. Administration of 68 μmol of calcium delays this drop and actually effects a slight increase in plasma calcium concentration at 1 hr. Five MRC units of hPTH-(1–34) plus 68 μmol of calcium effects an increase in plasma calcium of 1–2 mg/dl 1 hr after treatment. (MRC, Medical Research Council; for definition, see refs. 16 and 21.) There is a linear logarithmic dose–response curve for hPTH-(1–34) doses between 2 and 8 MRC units. Such a test is more sensitive than prior PTH *in vivo* bioassays and has been demonstrated to reflect primarily the action of PTH on bone (10). The use of the calcium-free diet for 1 week prior to experimentation enhances the sensitivity of the test system, as reported with PTH bioassays (17), while the coadministration of a small amount of calcium with hPTH-(1–34) in these experiments stabilized the assay by reducing the variance within groups (10).

Half the rats given hPTH-(1–34) plus $CaCl_2$ received the antagonist by tail-vein injection 30 sec before, immediately prior to, and 15 min after hPTH-(1–34) administration (Fig. 2). The injection volume and vehicle were the same as above. The remaining rats and all the controls received vehicle alone according to the same schedule. In different experiments, the antagonist dose was 180, 28, or 6 μg. In a second series of experiments, only the first two injections of antagonist were administered, each of 180 μg, and the 15-min injection was deleted. To determine whether the analog possessed agonist properties, it was given in one experiment to half the animals receiving $CaCl_2$ alone (without hPTH-(1–34), in a single dose equal to the maximum employed in the primary experiment. In a third series of experiments the $CaCl_2$ was omitted, to assure that the efficacy of the antagonist was independent of this additional calcium.

RESULTS

Treatment with 5 units of hPTH-(1–34) and 68 μmol of calcium increased plasma calcium 1.2 mg/dl over the response of controls who received calcium alone. Treatment with the antagonist, in which each of three injections represented 28 μg of peptide, abolished the PTH calcemic response and resulted in a serum calcium change that was indistinguishable from control rats receiving $CaCl_2$ alone (Fig. 3). The difference between the PTH-treated and antagonist- plus PTH-treated rats was statistically significant ($P < 0.001$). The analog had no agonist properties when administered at the maximum injection dose (180 μg) with 68 μmol of calcium (Table 1, treatment A). As expected, this higher dose of antagonist when administered as three individual injections also blocked the calcemic response to hPTH-(1–34). The animals treated with PTH and calcium were different from controls (vehicle and calcium) ($P < 0.02$), while animals treated with PTH, calcium, and antagonist were not (*P*, not significant) (Table 1, treatment B). Doses of antagonist representing only 6 μg were incapable of blocking the hPTH-(1–34) effects on plasma calcium (Table 1, treatment C).

Little is known about the degradation, disposal, or half-life of [Tyr^{34}]bPTH-(7–34)-amide *in vivo*. Therefore, in the first series of experiments, the antagonist was administered both before and after hPTH-(1–34) administration to maximize the changes of observing inhibitory effects. In the second series of experiments the third (15-min) injection was omitted (Table 1, treatment D). A statistically significant inhibitory effect was no longer seen, even when each of the first two injections represented 180 μg of antagonist. The difference between the groups given PTH and calcium and that given PTH, calcium, and antagonist was not significant, but the difference between the latter and control group (vehicle and calcium) was significant ($P < 0.01$).

In the third series of experiments (without calcium-containing vehicle), the plasma calcium of the vehicle-treated rats fell 0.2–0.4 mg/dl, while the plasma calcium of the PTH-treated rats rose 0.5–1.1 mg/dl—responses that were significantly different ($P < 0.01$). Treatment with the antagonist again prevented the PTH-induced increase in plasma calcium: the plasma calcium of rats receiving both the antagonist and PTH was not significantly different from that of rats receiving vehicle alone and was significantly lower than the plasma calcium of rats treated with PTH alone (Table 1, treatment E). These results are similar to those obtained with 68 μmol of calcium in the vehicle, indicating that the effects of the antagonist are not dependent upon any unrecognized peculiarity of assay design occasioned by the coadministration of calcium with the PTH.

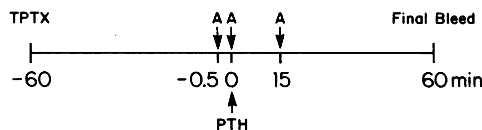

FIG. 2. Time line for experiments. Each intravenous injection of antagonist (A) was given over 10 sec. The second of these began 0.5 min after the first and was immediately followed by an intravenous injection of hPTH-(1–34) over 10 sec. A third injection of antagonist was given 15 min after the PTH in some experiments. Blood for plasma calcium determination was obtained immediately prior to the first injection and 60 min after the PTH injection. Control animals received vehicle on the same schedule, instead of antagonist and/or PTH. All animals were thyroparathyroidectomized (TPTX) 1 hr prior to administration of peptides or vehicle.

DISCUSSION

To our knowledge, this report is the first description of antagonism of the PTH-mediated calcemic response *in vivo* by a synthetic hormone analog. When administered intravenously in three consecutive doses that bracket in time the

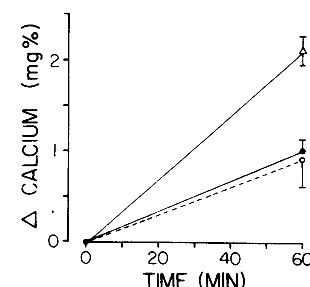

FIG. 3. Inhibition of PTH-mediated calcemic response by PTH analog [Tyr^{34}]bPTH-(7–34)-amide (antagonist). Animals received intravenous treatment with 68 μmol of $CaCl_2$ (○) (controls, $n = 11$); $CaCl_2$/5 MRC units of hPTH-(1–34) (△, $n = 11$); or $CaCl_2$/5 MRC units hPTH-(1–34)/28 μg of antagonist administered in three divided doses (●) (PTH and antagonist, $n = 12$). Values represent mean plasma calcium change from initial values ± SEM. ($P < 0.001$ for PTH vs. PTH and antagonist.)

Table 1. Effects of a parathyroid hormone antagonist on the hormone-mediated calcemic response in the presence or absence of coadministration of calcium

Treatment	Antagonist, μg	Injection frequency of antagonist	*n*	Pretreatment calcium, mg/dl	Posttreatment calcium, mg/dl	Δ Calcium, mg/dl
A. Ant/Ca	180	1	4	9.1	NS 10.3	1.2
Vehicle/Ca	—	—	5	9.2	10.2	1.0
B. PTH/Ca	—	—	10	9.75	† 11.9 *	2.2
PTH/Ant/Ca	180	3	11	9.7	NS 10.7	1.0
Vehicle/Ca	—	—	11	9.7	10.7	1.0
C. PTH/Ca	—	—	6	10.3	NS 12.1 †	1.8
PTH/Ant/Ca	6	3	6	10.0	† 11.9	1.9
Vehicle/Ca	—	—	6	10.0	10.8	0.8
D. PTH/Ca	—	—	5	9.5	NS 12.1 ‡	2.6
PTH/Ant/Ca	180	2	5	9.5	† 11.5	2.0
Vehicle/Ca	—	—	5	9.5	10.5	1.0
E. PTH	—	—	11	10.0	* 10.5 †	0.5
PTH/Ant	130	3	12	9.8	NS 9.8	0.0
Vehicle	—	—	13	9.9	9.5	−0.4

Ant, [Tyr[34]]bPTH-(7–34)-amide; PTH, hPTH-(1–34); vehicle, diluent; Ca, 68 μmol of calcium as $CaCl_2$; NS, not significant.

*$P < 0.02$; †$P < 0.01$; ‡$P < 0.001$; *P* values obtained by analysis of variance of posttreatment calcium values, with pretreatment calcium values used as a covariate, using Tukey's studentized range test for multiple comparisons.

administration of PTH, the analog [Tyr[34]]bPTH-(7–34)-amide (Fig. 1) inhibits rapid and bone dependent (10) PTH-stimulated increases in plasma calcium in thyroparathyroidectomized rats. Furthermore, this analog demonstrated no PTH-like agonist properties in these studies.

This investigation was undertaken after extensive structure–activity studies of PTH over a period of nearly a decade. The earlier studies generated fundamental principles for the design of PTH antagonists and led to the identification of [Tyr[34]]bPTH-(7–34)-amide as the first PTH antagonist effective *in vivo*. In addition, the present study serves to emphasize the critical importance of hormone assay design to successful evaluation of peptide hormone analogs.

Because supplies of the synthetic analog were limited, assays needed to be designed that minimized the quantity of antagonist required, that were highly sensitive to PTH, and that assessed rapidly occurring responses to the hormone. In addition, they must maximize the opportunity of observing inhibitory effects: i.e., the antagonist should be administered in molar excess over the agonist (PTH), and the antagonist should be favored in terms of timing of administration, relative to PTH, to permit the antagonist the greatest advantage over PTH in occupying receptors in target tissues *in vivo*.

In this report and in earlier studies (9, 10), assay systems were designed to satisfy these criteria and permit identification of antagonist properties for major parameters of PTH action *in vivo*. In the kidney-based assay, PTH stimulation of urinary cyclic AMP and phosphate excretion occur rapidly (minutes) and are presumed to be linked (1, 9).

In bone, the events following interaction of PTH with receptors on target cells may be more complex and generally require a longer time period (hours) for the bone cell-mediated calcium efflux to become evident than do the responses in kidney. To be effective at the level of bone *in vivo*, an antagonist might be required to possess relatively high affinity for bone receptors and/or to occupy these receptors for a longer period of time. Hence, the design of a PTH bioassay system that would reflect inhibitory effects on bone and the calcemic response was inherently more difficult and was delayed until a promising antagonist was identified.

The assay employed in this study uses animals whose response to PTH is enhanced because of acute thyroparathyroidectomy and prior dietary calcium restriction. In this assay, a single intravenous dose of 2–8 MRC units of hPTH-(1–34) produces a readily measured logarithmic dose calcemic response 1 hr later. The antagonist is given immediately prior to, simultaneous with, and 15 min after PTH administration so as to maximize the opportunity to inhibit hormonal action. At three repeated doses of antagonist, inhibition of the PTH–calcemic response was achieved.

Another element of assay design for PTH and this particular antagonist deserves emphasis. Omission of the last of the three antagonist doses (15 min after PTH administration) results in loss of inhibitory efficacy, even when the total dose of antagonist administered is six times greater than a dose that completely inhibits the PTH calcemic response. Clearly, additional pharmacokinetic studies are necessary to establish the optimal timing of the injection of the antagonist and the minimal doses of antagonist necessary to block completely the PTH-mediated responses. Certain data, such as the need for three injections of antagonist in the calcium elevation assay, suggest that the half-life of the antagonist *in vivo* is less than that of hPTH-(1–34). Data obtained *in vitro* in cell culture systems support the notion that the antagonist is rapidly degraded at a rate exceeding that of PTH (18). However, formal clearance studies are required to clarify this issue.

Although our calculations based on these studies indicate that a total molar ratio of antagonist to PTH of 300–1000:1 or higher may be required to inhibit PTH action, it is not clear whether the antagonist's avidity for PTH receptors or its pharmacokinetics determine its overall efficacy *in vivo*. Furthermore, different lots of PTH vary in their biopotency. As expected, when a more potent lot of PTH agonist was used in subsequent studies, changes in the amounts or relative amounts of antagonist and agonist were required to demonstrate inhibition of the PTH calcemic response. For these reasons, firm conclusions regarding the potency of the antagonist cannot be drawn from these experiments; rather, our studies were designed solely to demonstrate that antagonism of the PTH calcemic response can be achieved *in vivo*.

Although not ideally suited to determining potency of inhibitory analogs, the *in vivo* calcemic response assay (10) should facilitate an analog design program for PTH antagonists by permitting rapid screening of new analogs and identification of PTH antagonists. Finally, although *in vivo* antagonism of a PTH-mediated calcemic response is clearly demonstrated by our studies, it remains to be established if the analog can block PTH action under physiological condi-

tions or in chronic states of PTH-dependent hypercalcemia, such as experimental models of hyperparathyroidism (19). Antagonists that are more effective than [Tyr^{34}]bPTH-(7–34)-amide may be necessary for hormone action to be blocked continuously; the high doses of antagonist required in these studies may not be practical for either extended physiological studies or trials as a diagnostic or therapeutic agent in hypercalcemic disorders in humans. However, current results are encouraging. The analog [Tyr^{34}]bPTH-(7–34)-amide can antagonize the following three of the major parameters of PTH action *in vivo*: a PTH-mediated calcemic response, PTH-stimulated phosphaturia (9), and PTH-stimulated urinary cyclic AMP excretion (9).

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