Differential Effects of Parathyroid Hormone Responsive Cultured Human Cells on Biological Activity of Parathyroid Hormone and Parathyroid Hormone Inhibitory Analogues†

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ABSTRACT: We have employed parathyroid hormone (PTH) responsive human cells cultured from dermis or giant cell tumors of bone (GT) to evaluate the biological properties of a newly developed in vivo PTH inhibitor, [Tyr^{34}]bPTH-(7–34)-amide (PTH-Inh). Short periods of incubation of cells from dermis or GT with maximal stimulatory concentrations of PTH in the presence of increasing concentrations of PTH-Inh resulted in a dose-dependent inhibition of the adenosine cyclic 3′,5′-phosphate (cAMP) response (K_i = 3 × 10^{-7} M and 4.2 × 10^{-7} M for GT and dermal cells, respectively). In both cell cultures, PTH-Inh alone did not increase cAMP levels, and in desensitization experiments, preincubation with PTH-Inh alone did not desensitize cells to PTH. Hence, the analogue displayed no agonist properties. Unexpectedly, when PTH-Inh was incubated with dermal cells in the presence of PTH, the PTH-Inh failed to block desensitization, suggesting a loss of biological effectiveness of the inhibitor. When medium containing PTH-Inh alone was removed from dermal cells and tested for inhibition of the acute PTH response in untreated cells, there was apparent loss of inhibitory efficacy ($t_{1/2}$ = 20 h). In contrast, incubation of native PTH or bPTH-(1–34) with cells did not affect the biological activity of these ligands. Unlike the dermal cells, the PTH-Inh did block desensitization to PTH in GT, and there was no loss of inhibitor efficacy when medium containing PTH-Inh was incubated with GT (48 h) and then tested in untreated cells. To directly examine the effects of cells on these peptides, dermal cells were preincubated with PTH-Inh and biosynthetically ^3H-labeled bovine PTH. Analysis of these ligands by high-performance liquid chromatography revealed markedly different disappearance rates: by 20 h there was a 50% loss of PTH-Inh, whereas close to 100% of intact native hormone was still present. These findings indicate a preferential degradation of PTH-Inh over other PTH peptides as well as differences in hormone analogue degradation in PTH-responsive target tissues. The results of these studies have implications regarding the biological efficacy and metabolic stability of PTH analogues and provide direction for design of future inhibitory analogues of PTH.

In vitro and in vivo studies of the structure and activity of parathyroid hormone (PTH)[1] have demonstrated that the structural determinants necessary for full biological activity of this 84 amino acid polypeptide reside within the NH_2-terminal one-third (residues 1–34) of the native hormone (Tregear et al., 1973; Potts et al., 1982). Comparison of the properties of intact PTH with PTH fragments and analogues in different assay systems have defined within the amino acid sequence of the hormone separable domains responsible for receptor binding and biological activity (Goltzman et al., 1975; Rosenblatt et al., 1977; Nussbaum et al., 1980; Rosenblatt, 1981).

By employing a series of synthetic fragments containing NH_2-terminal deletions and a parathyroid hormone radioreceptor assay, the region 25–34 has been identified as the principal binding domain of the hormone (Nussbaum et al., 1980; Rosenblatt, 1981). It has also been determined that a small but critical message region (positions 1 and 2) is necessary for hormone action in vitro (Goltzman et al., 1975; Rosenblatt et al., 1977; Nussbaum et al., 1980; Rosenblatt, 1981).

These findings established a direction for the design of parathyroid hormone antagonists. An analogue of the 3–34 sequence of bovine PTH (bPTH), containing norleucine (Nle) and tyrosine (Tyr) substitutions, [Nle^8,Nle^{18},Tyr^{34}]bPTH-(3–34)-amide, was shown to be a true competitive inhibitor of PTH in vitro (Rosenblatt et al., 1977; Goldring et al., 1979). In other systems and in vivo, the substituted analogue demonstrated weak, but definite, PTH-like agonist properties (Segre et al., 1979; Martin et al., 1981; Horiuchi et al., 1983a,b). To eliminate residual agonist properties, the amino terminus was truncated beyond position 3. This modification resulted in the development of a new analogue, [Tyr^{34}]-bPTH-(7–34)-amide, which was recently shown to be an effective antagonist of PTH action in vivo. Coadministration of the analogue with PTH resulted in inhibition of PTH-induced excretion of urinary phosphate and adenosine cyclic 3′,5′-phosphate (cAMP) in rats (Horiuchi et al., 1983a,b) and inhibition of PTH-mediated increases in blood calcium in a rat-based bioassay. In contrast to studies with the 3–34-substituted PTH analogue, the 7–34 analogue was devoid of agonist properties (Horiuchi et al., 1983a,b).

To further investigate the biological properties and mechanisms of action of this PTH antagonist, we evaluated it in

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[1] Abbreviations: PTH, parathyroid hormone; bPTH, bovine parathyroid hormone; cAMP, adenosine cyclic 3′,5′-phosphate; GT, giant cell tumors of bone; Tyr, tyrosine; Nle, norleucine; PTH-Inh, [Tyr^{34}]-bPTH-(7–34)-amide; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; EDTA, ethylenediaminetetraacetate; PBS, phosphate-buffered saline; IBMX, 3-isobutyl-1-methylxanthine; HPLC, high-performance liquid chromatography; PGE_2, prostaglandin E_2.

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a cell culture model derived from human tissues. We previously showed that cells cultured from human giant cell tumors of bone and human dermis increase cAMP levels when incubated with PTH (Goldring et al., 1979). On the basis of PTH structure–function studies, desensitization experiments, and direct examination of PTH receptors by photoaffinity labeling techniques, the biological and physical properties of the PTH receptors in these cells are similar to those of the PTH receptors in other target tissues (Goldring et al., 1979, 1980, 1981, 1984). In this paper we show that the substituted PTH analogue [Tyr^{34}]bPTH-(7–34)-amide, when tested in the human cell cultures, behaves as a pure hormone antagonist, lacking agonist activity. In addition, we have characterized the biological properties of this new PTH antagonist and have evaluated its stability in our assay systems. Our results indicate that the synthetic analogue undergoes an accelerated loss of its biological efficacy compared to the biological activity of native hormone or synthetic bPTH-(1–34). These findings have significant implications in terms of the biological efficacy and metabolic stability of PTH antagonists and define new design strategies for development of future inhibitory analogues of PTH.

Materials and Methods

Tissue Preparation and Culture Procedures. Samples of human giant cell tumors of bone or human neonatal foreskin were prepared for culture by dispersion with trypsin–EDTA and clostridial collagenase, and cells were cultured in plastic dishes in Dulbecco's modified Eagle's medium (DMEM (Grand Island Biological Co., Grand Island, NY) plus 10% fetal calf serum (FCS (Microbiological Associates, Bethesda, MD), as previously reported (Goldring et al., 1979). After primary culture, cells were passaged into plastic dishes and maintained in DMEM/10% FCS. Forty-eight hours prior to hormone studies, cells were passaged at a density of 5×10^4 cells/well in plastic trays containing 24 individual wells.

Hormone Incubation. The design of each study is outlined in the description of individual experiments. In some studies, cAMP responses in cells were evaluated 48 h after passage into multiwell trays without prior exposure to hormone. For other studies, cells were cultured first in DMEM/10% FCS in the presence or absence of hormone. At the end of this preincubation, wells were washed 3 times with phosphate-buffered saline (PBS), and the cAMP response to fresh hormone was assessed by incubating cells for 20 min at 37 °C. Test incubation medium consisted of PBS supplemented with 0.9 mM magnesium, 0.25% bovine serum albumin (Pentex, Miles Laboratories, Kankakee, IL), 0.1% glucose, and 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Aldrich Chemical Co, Milwaukee, WI) plus or minus hormone. Incubations were terminated by freezing the trays in liquid N_2 (Goldring et al., 1979).

The effect of prior incubation with cells on the biological activities of PTH or the PTH analogue was also examined. For these studies, cells from human giant cell tumors of bone (GT) or dermis were cultured with medium (DMEM–10% FCS) containing native bPTH or the synthetic PTH analogues bPTH-(1–34) or PTH-Inh. At various time intervals, medium containing hormone was removed from wells and diluted 1:1 with test incubation buffer containing IBMX. This mixture (0.2 mL) was added to fresh cells (GT or dermis) that had been passaged 48 h earlier, and not previously exposed to hormone. Test incubations were performed as described previously. Matching samples of medium containing PTH or the synthetic PTH analogues were incubated in multiwell trays without cells and served as controls.

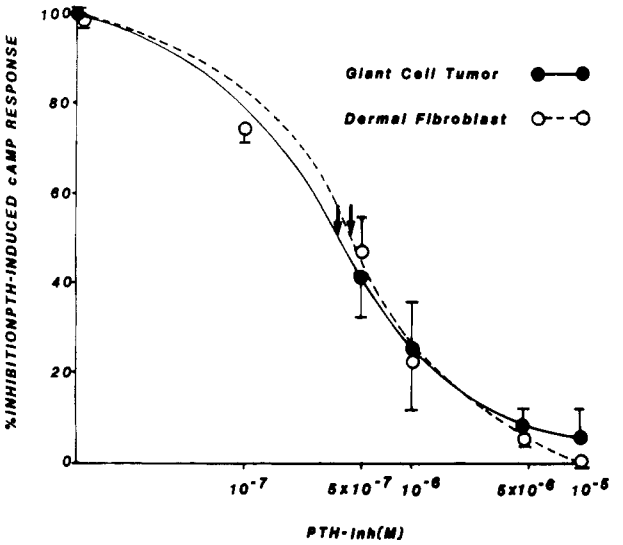

FIGURE 1: The levels of accumulated cAMP were measured in cells cultured from human giant cell tumor of bone (●) or dermis (○) after incubation for 20 min at 37 °C with bPTH-(1–84) 10^{-7} M, plus increasing concentrations of [Tyr^{34}]bPTH-(7–34)-amide (PTH-Inh). The concentrations of inhibitor that resulted in a 50% inhibition of PTH-induced increase in cAMP content in the two cell cultures are indicated by arrows and were 3.7×10^{-7} and 4.2×10^{-7} M in bone- and skin-derived cells, respectively. Values represent means ± SE for three samples.

In some experiments, biosynthetically labeled [[^{3}H]Tyr]-bPTH-(1–84) (approximately 1000 dpm/ng), prepared as previously described (Bringhurst et al., 1982), was added to medium containing PTH (10^{-7} M) or PTH-Inh (10^{-5} M) and incubated with cells or empty culture wells, as above. The mass of PTH contributed by the tracer was less than 1% of the total mass present. Aliquots withdrawn at various intervals were mixed with 0.2 volume of 60% acetonitrile/0.1% trifluoroacetic acid and stored at −20 °C pending subsequent analysis.

Chromatography. Samples of frozen medium were rapidly thawed, centrifuged at 10000*g* for 10 min, and analyzed directly by reverse-phase HPLC on a Bondapak C_{18} column (Waters Co., Milford, MA), essentially as previously described (Bringhurst et al., 1982) except that the hyperbolic gradient of 20–50% acetonitrile was elaborated over 20 rather than 15 min. Intact [^{3}H]PTH and PTH-Inh, eluting at 20 and 15 min, respectively, were quantitated by measurement of radioactivity and ultraviolet absorbance (206 nm), in the column effluent. Appropriate standards, dissolved in the culture medium (DMEM supplemented with 1% bovine serum albumin and 5 μg/mL transferrin), were employed to verify the elution positions of these peptides.

Results

To evaluate the effects of PTH-Inh on PTH-induced cAMP response, cells cultured from GT or dermis were incubated with bPTH-(1–84), 10^{-7} M, plus increasing concentrations of PTH-Inh. As shown in Figure 1, the concentrations of PTH-Inh that produced a 50% inhibition of the PTH-stimulated increase in cAMP content were 3.7×10^{-7} and 4.2×10^{-7} M in the GT and dermis derived cells, respectively. Cells from neither human tissue increased cAMP content when incubated with PTH-Inh alone at concentrations as high as 5×10^{-5} M (a concentration that produced nearly complete inhibition of the PTH-induced increase in cAMP levels). The specificity of the antagonism produced by the PTH-Inh was evaluated by incubating the human cells with prostaglandin E_2 (PGE_2) in the presence or absence of PTH-Inh. The

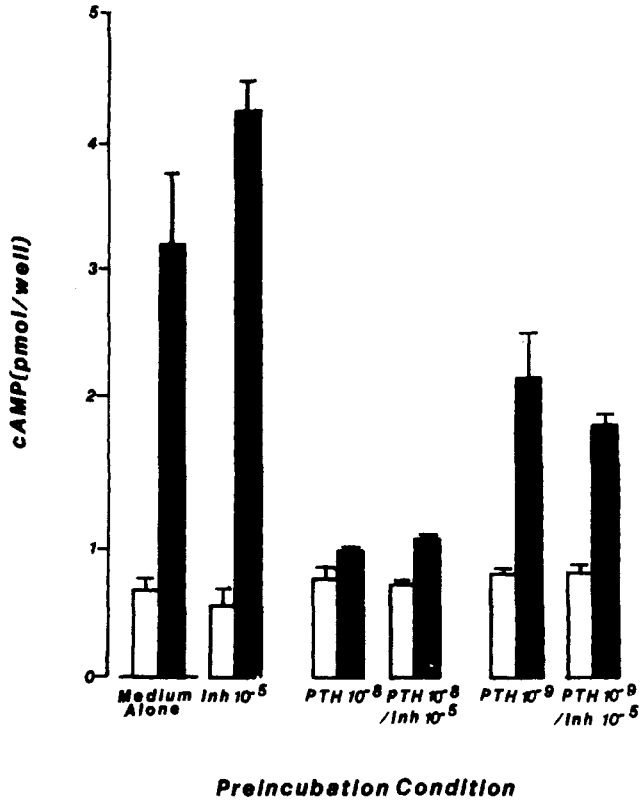

FIGURE 2: Cells cultured from human dermis were preincubated in DMEM/10% FCS alone (medium alone), bPTH-(1–84) alone (10^{-8} or 10^{-9} M), [Tyr^{34}]bPTH-(7–34)-amide alone (Inh, 10^{-5} M), or PTH plus inhibitor. After 48 h, cells were washed 3 times with PBS and test incubated for 20 min at 37 °C in buffer alone (□) or with PTH (10^{-7} M) (■) in the presence of IBMX (1 mM). The values represent means ± SE for three samples.

analogue did not significantly alter the PGE_2-stimulated increase in cAMP content (not shown).

In prior studies (Goldring et al., 1979, 1981), we showed that preincubation of cells cultured from GT or dermis with increasing concentrations of native PTH resulted in a dose-dependent loss of cAMP response when cells were retested with maximal stimulatory concentrations of PTH. The results of preincubation of cells cultured from human dermis with PTH-Inh are shown in Figure 2. As before, when cells were test incubated with PTH after 48-h incubation with PTH (10^{-8} or 10^{-9} M), there was a dose-dependent decrease in the magnitude of the PTH-induced increase in cAMP levels. We next tested the effects of preincubating cells with PTH in the presence of PTH-Inh. Although acute incubations of cells with PTH (at either concentration) in the presence of PTH-Inh (10^{-5} M) resulted in nearly 100% inhibition of the cAMP response (Figure 1), cells continuously cultured for 48 h in the presence of these ligands still exhibited desensitization to PTH.

To determine whether the failure of the PTH-Inh to block desensitization to PTH might be related to preferential loss of biological activity of the inhibitor during the preincubation period, the following studies were performed. Cells from human dermis were incubated with medium alone, bPTH-(1–84) alone, or bPTH-(1–84) plus PTH-Inh. At 0, 2, 6, and 24 h, medium was removed and mixed 1:1 with test-incubation buffer containing IBMX and then added to dermal cells passaged 48 h before and not previously exposed to hormone. As shown in Figure 3, the capacity of conditioned medium containing PTH to increase the cAMP levels in test cells did not change significantly despite exposure to cells for 24 h. In

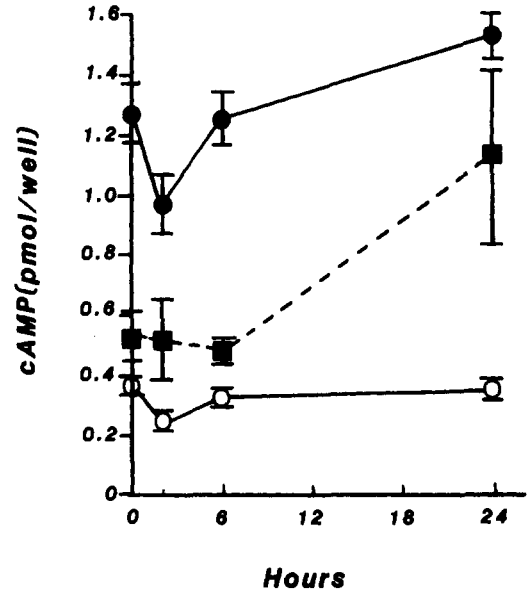

FIGURE 3: Cells from human dermis were incubated with medium alone, bPTH-(1–84) alone (10^{-7} M), or bPTH-(1–84) (10^{-7} M) plus PTH-Inh (10^{-5} M). At 0, 2, 6, and 24 h, medium was removed and mixed 1:1 with test-incubation buffer containing IBMX (1 mM) and then added to dermal cells not previously exposed to hormone. The levels of accumulated cAMP were measured after incubation for 20 min at 37 °C. Effects of the media are represented as follows: medium alone (○), PTH (●); PTH/PTH-Inh (■). Values represent means ± SE for three samples.

contrast, when medium containing bPTH-(1–84) plus PTH-Inh was removed and tested for its capacity to acutely increase cAMP levels, there was an apparent loss of biological efficacy of the PTH-Inh. When added to new cells, medium containing PTH plus PTH-Inh (at 0, 2, and 6 h) failed to increase cAMP response by the PTH analogue. In constrast, after 24-h exposure to cells, medium containing PTH plus PTH-Inh demonstrated greater than a 50% reduction in efficacy of the PTH-Inh when added to untreated cells. Similar results were observed in four additional studies (not shown).

To show that the apparent decrease in efficacy of the PTH-Inh was related to a cell-mediated process, medium containing PTH and the PTH analogue was incubated in parallel in the absence of cells. When this medium (after mixing 1:1 with test incubation buffer) was added to new cells, there was no change in the inhibitory capacity of the PTH-Inh (data not shown).

To determine whether the apparent susceptibility to loss of efficacy of the PTH-Inh after exposure to cells was related to the absence of the carboxy-terminal portion (portions 35–84) of the PTH molecule, synthetic bPTH-(1–34) alone or bPTH-(1–34) plus PTH-Inh was incubated in the presence or absence of dermal cells, and the conditioned medium was tested for biological activity in untreated cells. As shown in Figure 4, exposure of bPTH-(1–34) to cells for up to 41 h had no effect on the capacity of this ligand to increase cAMP levels when tested in untreated cells. In contrast, 24 h of exposure to cells of medium containing bPTH-(1–34) plus PTH-Inh resulted in a loss of efficacy of the PTH-Inh when tested on untreated cells. Loss of inhibitory efficacy occurred only when medium was incubated in the presence of cells.

Further evidence that exposure to dermal cells resulted in loss of biological activity of the inhibitor was obtained when cells were incubated with the inhibitory PTH analogue alone. This medium was removed from cells at 0, 17, 24, and 41 h and mixed with test-incubation buffer containing bPTH-(1–34). The PTH-Inh medium produced approximately a 40%

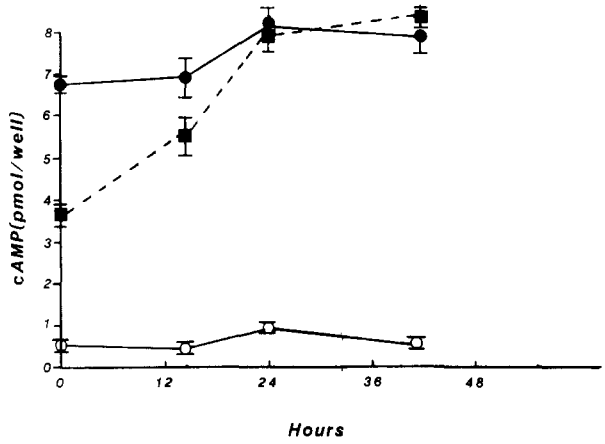

FIGURE 4: Cells from human dermis were incubated with medium alone, bPTH-(1–34) alone (10^{-7} M), or bPTH-(1–34) plus PTH-Inh (10^{-5} M). At 0, 17, 24, and 41 h, medium from cells incubated without hormone or with PTH were removed and mixed 1:1 with test-incubation buffer containing IBMX (1 mM) and then added to dermal cells not previously exposed to hormone. Medium from cells incubated with PTH-Inh was mixed 1:1 with buffer containing bPTH-(1–34) (10^{-7} M) and then added to new cells. The levels of accumulated cAMP were measured after incubation for 20 min at 37 °C. The effects of the media are represented as follows: medium alone (○), PTH (●); PTH-Inh plus PTH (■). Values represent means ± SE for three samples.

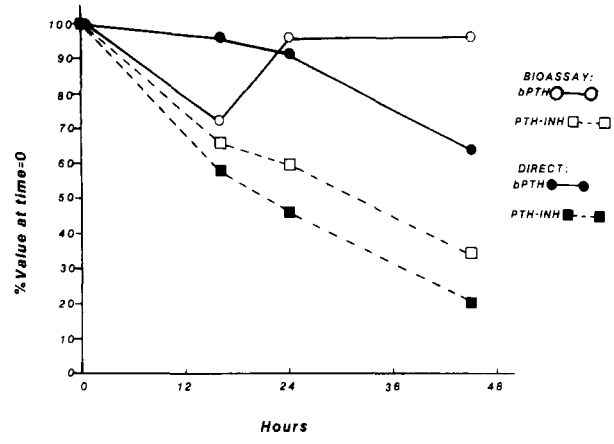

FIGURE 5: Cells from human dermis were incubated with medium containing bPTH-(1–84) (10^{-7} M) or PTH-Inh (10^{-5} M), and the levels of these peptides in samples removed at 0, 16, 24, and 45 h were assessed by both bioassay and chromatography. For bioassay, aliquots of medium were mixed 1:1 with test-incubation buffer containing IBMX (1 mM) and, in the case of the PTH-Inh media, fresh bPTH-(1–84), and the acute (20 min, 37 °C) cAMP responses of fresh dermal cells were measured in triplicate samples. The cAMP response to bPTH-(1–84)-containing media at $t = 0$ was 2.05 ± 0.36 pmol/well. The bioactivity of this medium at each time point is expressed as a percent of the value at time zero. The cAMP levels in cells tested at $t = 0$ with PTH-Inh media after being mixed with new bPTH-(1–84) was 0.92 ± 0.08 pmol/well. The inhibitory activity of the PTH-Inh-containing medium at each time point is expressed as a percentage of the activity at $t = 0$. Matching samples of medium were analyzed by reverse-phase HPLC, where the percentage of initial PTH-Inh (■) was quantitated directly by UV absorbance. Recovery of highly purified biosynthetically labeled [[^{3}H]Tyr]bPTH-(1–84) (●) was determined by measuring the percentage recovery of radioactivity in the appropriate region of the column effluent.

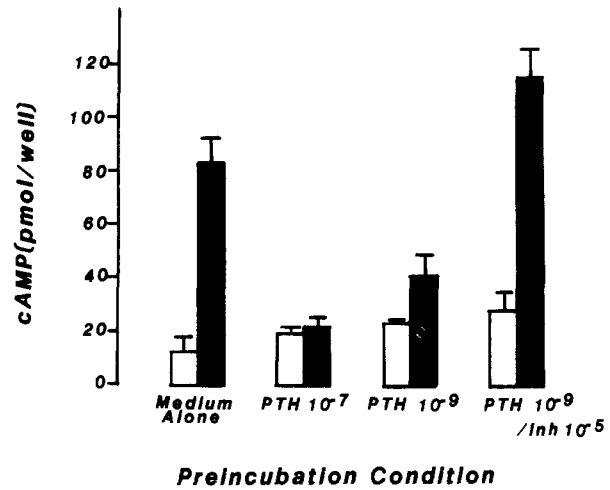

FIGURE 6: Cells cultured from GT were preincubated in DMEM/10% FCS alone (medium alone), bPTH-(1–84) alone (10^{-9} M), [Tyr^{34}]-bPTH-(7–34)-amide alone (Inh, 10^{-5} M), or PTH plus inhibitor. After 48 h, cells were washed 3 times with PBS and test incubated for 20 min at 37 °C in buffer alone (□) or with PTH (10^{-7} M) (■) in the presence of IBMX (1 mM). The values represent mean ± SE for three samples.

reduction in the cAMP response to PTH (6.93 ± 0.68 to 4.13 ± 0.22) at the early time points. However, at 41 h no inhibition of the PTH-induced cAMP response was detected (7.07 ± 0.50 compared to 8.40 ± 0.65).

The apparent preferential loss of inhibitory activity in the course of coincubations of PTH and the PTH analogue with dermal cells was further studied by serial chromatographic analysis of the levels of the intact PTH peptides in the culture medium, as described under Materials and Methods. In these experiments, concentrations of intact PTH analogue were determined by UV absorbance following isolation of the peptide from each sample of culture medium by reverse-phase HPLC. The fate of intact PTH was monitored directly in the same incubations with highly purified [^{3}H]bPTH-(1–84) as tracer in the culture medium. Assessment of intact PTH or the inhibitor directly or by assay of biological activity yielded comparable results as shown in Figure 5 and confirmed the accelerated disappearance of inhibitor compared to native hormone.

By direct measurement, 50% of the inhibitor originally present in the medium had disappeared by 20 h, at which time the level of intact PTH remained nearly equal to that present at the start of the incubations. By 48 h, less than 10% of the PTH analogue originally persent persisted in the medium, whereas the concentration of intact PTH was still maintained. In the case of [^{3}H]PTH, the loss of labeled intact hormone was accompanied by the appearance of earlier eluting labeled material consistent with generation of carboxy-terminal fragments of the hormone (Bringhurst et al., 1982). Numerous large peaks of absorbance eluting before 8 min precluded direct visualization of the products of PTH-Inh metabolism. These products would be expected to elute in this early region of the chromatogram.

To establish whether cells from other PTH-responsive tissues also have the capacity to alter the biological activity of the PTH-Inh, similar studies were performed in cells cultured from human GT. As shown in Figure 6, preincubation of GT with bPTH-(1–84) produced a dose-dependent loss in subsequent PTH-induced cAMP response. When GT was preincubated for 48 h with bPTH in the presence of PTH-Inh, desensitization to PTH was prevented. These results are different from those obtained in dermal cells (in which coincubation with PTH-Inh failed to block desensitization) and suggest that GT might interact with the PTH-Inh in a manner different from the dermal cells or GT may lack cell-based enzymes present in dermal cells that inactivate PTH-Inh.

To directly study the effect of GT on the efficacy of the PTH-Inh, GT was incubated with medium alone, bPTH-(1–84), PTH-Inh, or bPTH-(1–84) plus PTH-Inh. As shown in

Table I: Effects of Incubation of Cells Cultured from Human Giant Cell Tumor of Bone with PTH and PTH-Inh on the Biological Activity of PTH and PTH-Inh[a]

medium conditions	cAMP levels (pmol/well) after test incubation	
	−cells	+cells
medium alone	0.40 ± 0.12	0.57 + 0.08
+PTH (10^{-7} M)	1.89 ± 0.05	2.44 ± 0.50
+PTH-Inh (10^{-5} M)	0.37 ± 0.04	0.60 ± 0.08
+PTH (10^{-7} M)/PTH-Inh (10^{-5} M)	0.62 ± 0.06	0.67 ± 0.05

[a] Cells from GT were incubated with medium alone, bPTH, PTH-Inh, or PTH plus PTH-Inh. After 48 h, medium was removed and mixed 1:1 with test-incubation buffer containing IBMX (1 mM) and then added to GT not previously exposed to hormone. The levels of accumulated cAMP were measured after incubation for 20 min at 37 °C. Values represent mean ± SE for three samples.

Table I, when this conditioned medium was removed from GT after 48 h and tested in new GT after being mixed 1:1 with test-incubation buffer, inhibitory activity was still present. Cyclic AMP levels increased to 2.44 ± 0.50 pmol/well in cells after addition of the PTH-conditioned medium. In contrast to results in the dermal cell system, the medium containing PTH plus PTH-Inh failed to increase the cAMP levels above control (0.67 ± 0.05 and 0.57 ± 0.08, respectively), consistent with failure of these cells to alter the biological efficacy of the PTH-Inh.

Discussion

In this study we utilized cells derived from human tissues to investigate a newly developed inhibitory analogue of PTH, [Tyr^{34}]bPTH-(7-34)-amide. This cell culture model provided a system for characterizing and comparing the effects of PTH and PTH analogues in human cells of skeletal (giant cell tumor of bone) and dermal (neonatal foreskin fibroblast) (Goldring et al., 1979, 1980, 1981, 1984) origin. The results of PTH structure–function studies, desensitization experiments, and direct examination of PTH receptors by photoaffinity labeling techniques demonstrated that the PTH receptors in these cells display characteristics similar to the PTH receptors in other tissues, such as kidney (Goldring et al., 1984). The in vitro systems offer advantages in terms of ease of performance of assays and requirements for minimal amounts of synthetic PTH analogues.

Using cell culture systems, we evaluated the biological properties of the analogue [Tyr^{34}]bPTH-(7–34)-amide in vitro and compared these results to observations previously made in vivo. When tested in the two in vitro cell culture systems, the antagonist analogue demonstrated no PTH-like agonist properties. The in vitro inhibitory activity was comparable to that observed in vivo: a ratio of inhibitor to PTH of approximately 100:1 was required to achieve complete inhibition of PTH action.

Additional evidence for lack of agonist activity in the PTH analogue is provided by the desensitization experiments. In previous studies (Goldring et al., 1981), we found that desensitization is a very sensitive indicator of PTH agonist activity. Preincubation of cells with concentrations of bPTH as low as 10^{-10} M resulted in as much as a 20–30% reduction in the magnitude of the PTH-induced cAMP response when cells were retested with this hormone. When cells were preincubated with the PTH-Inh, no subsequent decrease in the PTH-induced cAMP response was observed, consistent with the lack of an agonist effect. Evaluation of the analogue in vitro, however, did lead to a series of unexpected observations. The preincubation studies with PTH plus PTH-Inh revealed that the PTH analogue failed to block desensitization to PTH despite continuous presence of the inhibitor at concentrations that produced (in short-term incubation) complete antagonism of the PTH-stimulated increase in cAMP levels. These results are in contrast to those obtained earlier with a different substituted PTH inhibitory analogue, [Nle^{8},Nle^{18},Tyr^{34}]-bPTH-(3–34)-amide. Incubation of the latter analogue with PTH partially blocked desensitization to PTH (Goldring et al., 1981).

Our present studies provide possible explanations for the failure of [Tyr^{34}]bPTH-(7–34)-amide to block desensitization to PTH. After 48-h incubation with cells, medium containing the PTH-Inh plus PTH failed to exhibit any inhibitory activity when retested on untreated cells. Similarly, after a 48-h exposure to cells, medium containing only the PTH-Inh when mixed with PTH and tested on untreated cells was also devoid of inhibitory activity.

Several possible mechanisms might account for the loss of inhibitory activity observed during exposure of the PTH analogue to dermal cells: (a) cell-specific metabolism of the PTH-Inh to a biologically inactive species, b) uptake of the ligand by cells, with consequent decrease in medium concentration, or (c) stimulation by PTH of cellular mechanisms or products that could interfere or compete with the biological effects of the analogue. The cell-associated loss of analogue inhibitory activity in the absence of PTH argues against mechanism c. Our further observation that the rate of loss of intact PTH-Inh measured by direct chromatographic analysis closely parallels the loss of inhibitory potency measured biologically strongly suggests that preferential metabolism or degradation of the inhibitor is responsible for this phenomenon in the dermal–cell system. Overall, the results of our comparative studies of the biological activities of bPTH-(1–84), bPTH-(1–34), and PTH-Inh in this system indicate a marked reduction in stability that is unique to the inhibitory analogue and suggest that deletions at the NH_2 terminus may lead to accelerated loss of activity during exposure to cells.

Numerous studies of the metabolism of PTH and its potential physiologic importance have been published (Segre et al., 1972, 1974, 1976, 1977, 1981a,b; Hruska et al., 1975; Neuman et al., 1975; Bringhurst et al., 1982). These studies suggest that the liver and, to a lesser extent, the kidneys are the major organs responsible for the clearance of intact PTH from the plasma. Little is known regarding the metabolism of hormone by other target tissues such as bone or the metabolism of PTH analogues. Prior studies suggest that the metabolism of intact PTH is accompanied by the generation of predominantly COOH-terminal PTH fragments, consistent with the effects of endopeptidases that cleave the molecule between residues 33 and 34, 36 and 37, and 40 and 41. The demonstration of metabolism of biologically active, unmodified, radiolabeled bPTH by isolated rat Kupffer cells (Segre et al., 1981a,b; Bringhurst et al., 1982) as well as in vivo studies in nephrectomized rats suggest that hepatic macrophages generate the majority of circulating COOH-terminal metabolites of PTH. It is unlikely that macrophages present as cellular contaminants in our cell culture systems could account for the data on analogue inactivation that we obtained. The dermal cells used in our studies have been maintained in monolayer culture for several years and passaged greater than 50 times. In addition, these cells lack macrophage surface markers. Furthermore, the degradative process occurring in the dermal cell system may be different in nature from that mediated by hepatic macrophages and kidney in vivo. The increased stability of [Nle^{8},Nle^{18},Tyr^{34}]bPTH-(3–34)-amide over

[Tyr^{34}]bPTH-(7–34)-amide suggests a possible role for amino exopeptidases in the degradation and inactivation of the 7–34 antagonists.

The methods used in the present studies did not permit precise analysis of the cleavage sites that presumably are responsible for the disappearance of intact PTH inhibitor. Nevertheless, if a process such as amino-terminal digestion does occur in these cell cultures, then the longer 3–34 analogue might withstand amino-terminal truncation at least to position 7 without complete loss of inhibitory efficacy.

While the effect of truncation beyond position 7 on inhibitory potency is not known and can only be speculated upon, rapid decreases in PTH receptor avidity for shortened fragments have been observed (Nussbaum et al., 1978) and would suggest that amino-terminal digestion beyond position 7 might produce analogues lacking significant inhibitory potency. Doppelt, Rosenblatt, and Neer (unpublished data), have studied the effects of the analogue on the PTH-mediated calcemic response in vivo and have recently obtained data suggesting that the half-life for the analogue in vivo is very short (minutes). This is significantly less than the half-life for bioactivity of bPTH-(1–34) in vivo (Doppelt et al., 1983).

In contrast to results obtained with dermal cells, the cells cultured from the giant cell tumor, which possess PTH receptors indistinguishable from those in the dermal cells, failed to diminish the inhibitory activity of the PTH-Inh. These results are consistent with the presence of certain enzymes with the capacity to degrade the PTH analogue in the dermal system that are lacking in the bone-derived cell cultures. Alternatively, differential mechanisms for the interaction of the PTH-Inh and PTH may exist in these two PTH-responsive target tissues. These differential effects may have significance in terms of the biological efficacy of the inhibitor in different target organs in vivo.

In summary, we have shown that an inhibitory analogue of PTH is less stable than native bPTH or synthetic bPTH-(1–34) of sequence length comparable to the analogue. It is possible that the susceptibility to inactivation displayed by the analogue in vitro may have significance ultimately in terms of in vivo effectiveness of this hormone antagonist. Therefore, these studies have implications for the design of potential PTH antagonists. Studies are under way in our laboratory to define the specific mechanisms involved in loss of biological activity and the chemical nature of the metabolites of the analogue that are produced. On the basis of the results of such studies, we plan to incorporate structural modifications in PTH-Inh to generate more stable PTH antagonists that maintain inhibitory efficacy both in vitro and in vivo.

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