Minimization of Parathyroid Hormone

NOVEL AMINO-TERMINAL PARATHYROID HORMONE FRAGMENTS WITH ENHANCED POTENCY IN ACTIVATING THE TYPE-1 PARATHYROID HORMONE RECEPTOR*

Received for publication, December 14, 1999, and in revised form, April 17, 2000

Masaru Shimizu, John T. Potts, Jr., and Thomas J. Gardella‡
From the Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

The amino-terminal and carboxyl-terminal portions of the 1–34 fragment of parathyroid hormone (PTH) contain the major determinants of receptor activation and receptor binding, respectively. We investigated how the amino-terminal signaling portion of PTH interacts with the receptor by utilizing analogs of the weakly active fragment, rat (r) PTH(1–14)NH₂, and cells transfected with the wild-type human PTH-1 receptor (hP1R-WT) or a truncated PTH-1 receptor which lacked most of the amino-terminal extracellular domain (hP1R-delNt). Of 132 mono-substituted PTH(1–14) analogs, most having substitutions in the (1–9) region were inactive in assays of cAMP formation in LLC-PK1 cells stably expressing hP1R-WT, whereas most having substitutions in the (10–14) region were active. Several substitutions (e.g. Ser³ → Ala, Asn¹⁰ → Ala or Gln, Leu¹² → Arg, Gly¹⁵ → Ala, His¹⁴ → Trp) enhanced activity 2–10-fold. These effects were additive, as [Ala³,¹⁰,¹²,Arg¹¹,Trp¹⁴]rPTH(1–14)NH₂ was 220-fold more potent than rPTH(1–14)NH₂ (EC₅₀ = 0.3 ± 0.1 and 135 ± 16 μM, respectively). Native rPTH(1–11) was inactive, but [Ala³,¹⁰,¹²,Arg¹¹]rPTH(1–11)NH₂ achieved maximal cAMP stimulation (EC₅₀ = 17 μM). The modified PTH fragments induced cAMP formation with hP1R-delNt in COS-7 cells as potently as they did with hP1R-WT; PTH(1–34) was 6,000-fold weaker with hP1R-delNt than with hP1R-WT. The most potent analog, [Ala³,¹⁰,¹²,Arg¹¹,Trp¹⁴]rPTH(1–14)NH₂ stimulated inositol phosphate production with hP1R-WT. The results show that short NH₂-terminal peptides of PTH can be optimized for considerable gains in signaling potency through modification of interactions involving the regions of the receptor containing the transmembrane domains and extracellular loops.

In mammals, parathyroid hormone (PTH)¹ plays a vital role in regulating blood calcium concentrations, and PTH-related peptide (PThrP) plays a critical role in the development of the fetal skeleton (1). The biological actions of both of these peptides are mediated by the PTH/PThrP receptor (or PTH-1 receptor) (2), a family B G protein-coupled receptor (3) that strongly activates the adenylyl cyclase/protein kinase A-signaling cascade (2), and more weakly the phospholipase C protein kinase C-signaling pathway (4). The mechanisms by which parathyroid hormone and PThrP bind to the PTH-1 receptor and induce receptor activation are poorly understood but appear to involve multiple sites of molecular interaction. Early studies of PTH fragment analogs assigned the major determinants of receptor-binding affinity and cAMP-stimulating potency to the COOH-terminal and NH₂-terminal portions of the fully active PTH(1–34) peptide, respectively (5, 6). PTH(1–34)-based analogs with NH₂-terminal deletions, such as PTH(3–34) and PTH(7–34), bind efficiently to the receptor and are severely defective in stimulating a cAMP response; such peptides thus function as PTH-1 receptor antagonists (7–9). The dominant role that the NH₂-terminal residues of PTH and PThrP play in receptor activation is further reflected by their high level of evolutionary conservation.

The anabolic effects of PTH on bone density (10, 11) have prompted considerable interest in the development of new PTH-1 receptor agonist analogs. Recently PTH(1–28) was shown to be an effective agonist for cAMP production in cell-based assays, although potency was ~10-fold reduced from that of PTH(1–34) (12, 13). Recently we found that in COS-7 or LLC-PK1 cells transfected with high levels of rat or human PTH-1 receptors, a fragment as short as PTH(1–14) elicited ~20-fold increases in cAMP formation levels (14). Although the potency of PTH(1–14) in these transfected cells was weak compared with PTH(1–34) (EC₅₀ = 1 nM and 100 μM, respectively), the response was sufficient for us to perform an initial structure-activity relationship analysis. In this previous study, we found that most alanine substitutions in the (1–9) region severely diminished PTH(1–14)-signaling activity, whereas alanine substitutions in the (10–14) region preserved activity (14). We also showed that PTH(1–14) could activate a truncated PTH receptor that lacked most of the NH₂-terminal domain (14). These studies with PTH(1–14) and the truncated receptor were consistent with the hypothesis suggested by other mutational and cross-linking data (15–19) that residues in the NH₂-terminal portion of PTH(1–34) interact with the region of the receptor containing the seven transmembrane domains and extracellular loops. Other peptide hormones that bind family B receptors, such as calcitonin, secretin, and glucagon, and are comparable in size to PTH(1–34) may utilize a similar topological arrangement in binding to their cognate receptors (20, 21); however, small NH₂-terminal activating peptides for these other family B receptors have thus far not been reported.

In the current study, we use PTH(1–14) as a starting scaffold for investigating whether amino acid modifications can be identified that both enhance the signaling potency of PTH(1–14) and enable further reductions in agonist peptide length. The results show that the NH₂-terminal residues of PTH can be optimized, in that greater agonist potency can be achieved in peptides as short as 11 amino acids. Such minimized peptides serve as useful probes of the receptor-interaction mechanism.

* This work was supported by National Institutes of Health Grant DK-11794. The costs of publication of this article were deferred in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 617-726-3683; Fax: 617-726-7543; E-mail: gardella@helix.mgh.harvard.edu.

¹ The abbreviations used are: PTH, parathyroid hormone; r, rat; h, human; PThrP, PTH-related peptide; Fmoc, N-(9-fluorenyl)methoxycarbonyl; HPLC, high performance liquid chromatography.

This paper is available on line at http://www.jbc.org
as we show that the activity-enhancing effects of the ligand modifications are mediated through the portion of the receptor containing the seven transmembrane domains and extracellular loops.

**MATERIALS AND METHODS**

**Peptides**—The Massachusetts General Hospital Biopolymer Synthesis Facility (Boston, MA) prepared all peptides used in this study. Each peptide contained a carboxyl-terminal amide and a free amino group at the amino terminus, except for the PTH(1–14) analogs desN and desN-A, which contained the position 1 modifications of desmethionine and desamino, respectively, to assay. At the time of the assay, the cells were incubated with binding buffer containing LiCl (30 mM) and treated with the same buffer with or without a PTH analog. The cells were then incubated at 37 °C for 40 min, after which the buffer was removed and replaced by 0.5 ml of ice-cold 5% trichloroacetic acid solution. After 3 h on ice, the lysate was collected and extracted twice with ethyl ether. The total inositol phosphates were eluted as described previously (30), and counted in liquid scintillation mixture.

**Data Calculation**—Calculations were performed using Microsoft Excel. Nonlinear regression analysis of cAMP stimulation data was performed using the following equation: \( y = \frac{E_{\text{max}}}{(1 + x/E_{\text{EC}_{50}})} \), where the initial parameter values were estimated from the primary data, and the Excel “solver function” was then used to vary the four parameters in order to minimize the differences between the predicted and actual responses (least-squares method) (31). For each experiment, the maximally constrained to within ±1 standard deviation of the maximum response observed in that experiment for rPTH(1–34) at a dose of 1 × 10–7 M. The optimized equations were used to curve-fit the data shown in the graphs and to obtain the E_{\text{max}} and corresponding maximum (E_{\text{max calorie}}) values reported in the tables. The observed maximum responses (E_{\text{max calorie}}) were those attained by each NH2-terminal fragment analog at a dose of 100 µM, and by each PTH(1–34) analog at a dose of 100 nm, except for studies in cells expressing hP1R-delNT where the E_{\text{max calorie}} for rPTH(1–34) and hPTH(1–34) was determined at a dose of 10 µM and for [Ala\textsubscript{1},3,9,12,Arg\textsubscript{11},Tyr\textsubscript{34}]hPTH(1–34)NH\textsubscript{2} at a dose of 20 µM. In some cases where the dose-response curves did not attain a true asymptotic maximum, as with native rPTH(1–14), the E_{\text{max calorie}} values are greater than the E_{\text{max calorie}} values. The statistical significance between two data sets was determined using a one-tailed Student’s t test assuming unequal variances for the two sets.

**RESULTS**

**PTH(1–14) Analogs in HKRK-B7 Cells—PTH(1–14) analogs having single substitutions (152 total) were tested for the ability to stimulate cAMP formation in HKRK-B7 cells. The substitutions were chosen such that at least one of each type of the 20 natural amino acids was introduced at each position, thus enabling a comparison of the effects of varied side chain chemistries (e.g. size, polarity, ionic charge, hydrophobicity, aromaticity, and proline) on receptor activation. The analogs and the control peptide (native rPTH(1–14)NH\textsubscript{2}) were tested at a single dose of 100 µM; rPTH(1–34) was tested at a maximum stimulatory dose (10–7 M). Both assays showed in Fig. 1, native rPTH(1–14) and rPTH(1–34) stimulated 28- and 58-fold increases in cAMP formation, respectively, as compared with the cAMP level in unstimulated cells, which was less than 6 pmol/ well. This response range ensured that both activity-enhancing and activity-impairing effects could be readily detected in the PTH(1–14) analogs. As shown in Fig. 1, most substitutions in the (1–9) segment of rPTH(1–14) severely reduced signaling activity; only positions 1 and 3 were partially tolerant to substitution, and the Ser\textsuperscript{4} → Ala substitution resulted in an enhancement in activity. The substitution of leucine 7 with Phe also preserved activity, a result that correlates with the occurrence of Phe at position 7 in bovine PTH. Substitutions in the (10–14) region had markedly less severe effects on cAMP-signaling function,
Effects of single substitutions in PTH(1–14) on cAMP responses in LLC-PK1 cells stably transfected with the PTH-1 receptor.

Nativel rat PTH(1–14)NH₂ (native) and 132 different monosubstituted analogs of that peptide (indicated on the figure key) were tested at a dose of 100 nM for the ability to stimulate cAMP accumulation in the stably transfected LLC-PK1-derived cell line, HKKK-B7 (~950,000 hPTH-1 receptors/cell). The data (mean ± S.E.) were combined from two separate experiments in which each peptide was assayed in duplicate. The maximum cAMP response obtained in these assay with rPTH(1–34) (100 nM) was 221 ± 6 pmol/well.

Fig. 1. Effects of single substitutions in PTH(1–14) on cAMP responses in LLC-PK1 cells stably transfected with the PTH-1 receptor.

Native rat PTH(1–14)NH₂ (native) and 132 different monosubstituted analogs of that peptide (indicated on the abscissa) were tested at a dose of 100 nM for the ability to stimulate cAMP accumulation in the stably transfected LLC-PK1-derived cell line, HKKK-B7 (~950,000 hPTH-1 receptors/cell). The data (mean ± S.E.) were combined from two separate experiments in which each peptide was assayed in duplicate. The maximum cAMP response obtained in these assay with rPTH(1–34) (100 nM) was 221 ± 6 pmol/well.

Fig. 2. Dose-response analysis of PTH analogs in LLC-PK1 cells stably transfected with the PTH-1 receptor. The control peptide, [Nle³,²¹,Tyr³⁴]rPTH(1–34)NH₂ (rPTH (1–34)) and the native or modified NH₂-terminal fragment analogs of rPTH(1–14)NH₂ were tested at varying doses for cAMP-stimulating activity in HKKK-B7 cells. Shown are data (mean ± S.E.) combined from three experiments, each performed in duplicate. The symbols are defined in the figure key, and the curves were fit to the data points using non-linear regression analysis, as described under “Experimental Procedures.” Single-letter amino acid codes are used.

as compared with those in the (1–9) region, and a number of activity-enhancing substitutions were found. Dose-response analysis of peptides containing some of these enhancing substitutions indicated that cAMP-signaling potency was improved from 2.4-fold (Ser³ → Ala) to 9.7-fold (Leu¹¹ → Arg), relative to native rPTH(1–14) (Table I). Several of the activity-enhancing substitutions were then combined to yield PTH(1–14) analogs with two or more modifications (Table I). In most cases, the effects of these substitutions on activity were additive, as the potency of the peptides tended to improve as the substitutions were combined. The most potent peptides in the series were those containing four or five substitutions, such as [Ala³,⁴,¹⁰,¹₂,Arg¹¹,Trp¹⁴]rPTH(1–14)NH₂, which exhibited an EC₅₀ that was 220-fold lower than that of rPTH(1–14) (EC₅₀ = 0.6 ± 0.1 µM and 133 ± 16 µM, respectively) (Table I). As shown in Fig. 2 and Table I, the above activity-enhancing substitutions improved the cAMP-signaling capability of shorter length PTH peptide fragments that were previously found to be inactive (14). In fact, [Ala³,⁴,¹₀,⁰,Arg¹¹]rPTH(1–11)NH₂ was more potent than native rPTH(1–14) in stimulating a cAMP response in HKKK-B7 cells (EC₅₀ = 17.1 ± 0.7 µM and 133 ± 16 µM, respectively; p < 0.0001), and elicited a maximum that was comparable to that observed for rPTH(1–34) (Fig. 2). A modest level of agonist activity could be detected

### Table I

| Peptide                        | EC₅₀ (µM) | EC₅₀(calc) (µM) | EC₅₀(max) (µM) | n   |
|-------------------------------|----------|-----------------|----------------|-----|
| [Nle³,²¹,Tyr³⁴]rPTH(1–34)NH₂   | 3.5 ± 0.5 | 302 ± 28        | 294 ± 28       | 13  |
| [Ala³,¹²,Arg¹¹,Tyr³⁴]rPTH(1–34)NH₂ | 1.3 ± 1.1 | 305 ± 69        | 293 ± 48       | 4   |
| rPTH(1–14)NH₂ (native)         | 133 ± 16 | 299 ± 27        | 124 ± 6        | 10  |
| [Ala³]rPTH(1–14)NH₂            | 54.3 ± 17| 341 ± 64        | 233 ± 12       | 3   |
| [Ala³]rPTH(1–14)NH₂            | 39.6 ± 11| 316 ± 45        | 252 ± 14       | 3   |
| [Arg³]rPTH(1–14)NH₂            | 13.7 ± 5.4 | 344 ± 64        | 257 ± 23       | 4   |
| [Ala³]rPTH(1–14)NH₂            | 13.8 ± 2.6 | 320 ± 47        | 299 ± 26       | 3   |
| [Ala³,¹⁰]rPTH(1–14)NH₂         | 69.6 ± 9.9 | 381 ± 20        | 238 ± 3        | 3   |
| [Ala³]rPTH(1–14)NH₂            | 13.0 ± 1.9 | 357 ± 5         | 316 ± 7        | 3   |
| [Ala³]rPTH(1–14)NH₂            | 26.9 ± 4.6 | 357 ± 5         | 294 ± 12       | 3   |
| [Ala³]rPTH(1–14)NH₂            | 7.5 ± 1.8 | 382 ± 16        | 337 ± 21       | 3   |
| [Ala³,¹⁰]rPTH(1–14)NH₂         | 20.4 ± 4.0 | 390 ± 19        | 344 ± 11       | 3   |
| [Arg³,¹⁰]rPTH(1–14)NH₂         | 4.8 ± 1.7 | 389 ± 22        | 364 ± 11       | 3   |
| [Ala³,¹⁰]rPTH(1–14)NH₂         | 4.3 ± 0.7 | 295 ± 12        | 255 ± 14       | 3   |
| [Ala³,¹⁰]rPTH(1–14)NH₂         | 15.4 ± 0.4 | 246 ± 14        | 212 ± 5        | 3   |
| [Ala³,¹⁰]rPTH(1–14)NH₂         | 3.4 ± 0.7 | 264 ± 7         | 259 ± 10       | 3   |
| [Ala³,¹⁰,¹²]rPTH(1–14)NH₂      | 1.3 ± 0.2 | 252 ± 12        | 254 ± 14       | 3   |
| [Ala³,¹⁰,¹²]rPTH(1–14)NH₂      | 0.83 ± 0.22 | 264 ± 16        | 269 ± 10       | 7   |
| [Ala³,¹⁰,¹²]rPTH(1–14)NH₂      | 0.79 ± 0.19 | 235 ± 3         | 233 ± 3        | 3   |
| [Ala³,¹⁰,¹²]rPTH(1–14)NH₂      | 0.57 ± 0.11 | 262 ± 9         | 273 ± 14       | 3   |
| Shorter length analogs        |          |                 |                |     |
| [Ala³]rPTH(1–10)NH₂           | 770 ± 139| 250 ± 13        | 73 ± 6         | 3   |
| [Ala³,¹⁰]rPTH(1–11)NH₂         | 17.1 ± 0.7 | 248 ± 13        | 220 ± 10       | 3   |
| [Ala³,¹⁰,¹²]rPTH(1–12)NH₂      | 10.2 ± 0.2 | 238 ± 9         | 229 ± 9        | 3   |
| [Ala³,¹⁰,¹²]rPTH(1–13)NH₂      | 2.4 ± 0.3  | 239 ± 7         | 235 ± 8        | 3   |

Peptides were evaluated for dose-dependent stimulation of cAMP formation in HKRK-B7 cells; the EC₅₀ and corresponding E₅₀(calc) values were calculated using nonlinear regression analysis, as described under “Materials and Methods.” The maximum response observed (E₅₀(max)) for [Nle³,²¹,Tyr³⁴]rPTH(1–34)NH₂ (100 µM) and each NH₂-terminal fragment analog (100 µM) is also shown. Note that in each experiment, the E₅₀(calc) value for each peptide was constrained to within ±1 standard deviation of the E₅₀(max) value for that experiment, and that for [Nle³,²¹,Tyr³⁴]rPTH(1–34)NH₂, which ranged in these experiments from 170 to 430 pmol/well. Thus, for some weaker fragment analogs, such as native rPTH(1–14), the E₅₀(calc) value is less than the corresponding E₅₀(max) value. Values are means (± S.E.) for the number of experiments indicated (n).
with [Ala₃,Gln¹⁰]rPTH (1–10)NH₂, but this peptide was still weaker than native PTH (1–14).

**ROS-17/2.8 Cells**—We examined several of the peptides for activity in the rat osteosarcoma cell line ROS 17/2.8 (24) as a means to assess analog effects in a commonly used cell line that is believed to be representative of osteoblasts. These cells express a relatively low level of endogenous PTH-1 receptors (~70,000/cell). The analog [Ala₃,¹⁰,¹²,Arg¹¹]rPTH(1–14)NH₂ was a full agonist for cAMP production in these cells, and was 71-fold more potent than native rPTH(1–14)NH₂ (EC₅₀ = 5.6 ± 2.5 μM and 400 ± 64 μM, respectively; Fig. 3 and Table II). Native rPTH(1–11) was inactive (data not shown) but [Ala₃,¹⁰,¹²,Arg¹¹]rPTH(1–11)NH₂ (EC₅₀ = 120 ± 41 μM) was 3.3-fold more potent than native PTH (1–14) (p < 0.03). The NH₂-terminal peptides were generally 5–7-fold weaker in ROS 17/2.8 than they were in HKRK-B7 cells, while the PTH (1–34) analogs were ~7–15-fold more potent in ROS 17/2.8 cells than they were in HKRK-B7 cells. The relative order of potencies observed for the native and modified truncated analogs in the osteosarcoma cells closely followed that seen in the transfected LLC-PK1 cells.

**A Truncated PTH-1 Receptor in COS-7 Cells**—To determine whether the enhancing effects of the substitutions in the PTH (1–14) region could be attributed to interactions involving the receptor’s large (~170-amino acid) amino-terminal extracellular domain, we tested several of the analogs for activity in COS-7 cells transiently transfected with a truncated rPTH-1 receptor that lacks most of this domain (hP1R-delNt). The control peptide [Tyr³⁴]hPTH(3–34)NH₂ was ~54,000-fold less potent than hP1R-delNt (Table I). A similarly diminished potency was observed for rPTH(1–34) in cells expressing hP1R-delNt (Table III). Such markedly reduced potencies for PTH(1–34) analogs were observed previously in our studies on a similarly truncated rat PTH-1 receptor (14) and highlight the importance of the receptor’s NH₂-terminal domain for efficient interaction with intact PTH (1–34) (32, 33). In contrast to these severe effects on PTH (1–34) potency, the potency of native rPTH (1–14) was largely unaffected by the deletion of the NH₂-terminal domain (Fig. 4, A and B; Table III). With both hP1R-delNt and hP1R-WT, [Ala₃,¹⁰,¹²,Arg¹¹]rPTH(1–14)NH₂ was ~100-fold more potent in stimulating cAMP formation than was native rPTH (1–14) (Table III).

Introduction of the activity-enhancing substitutions into hPTH(1–34) produced an analog, [Ala₃,¹⁰,¹²,Arg¹¹,Tyr³⁴]hPTH(1–34)NH₂, that exhibited only a modestly improved potency, as compared with [Tyr³⁴]hPTH(1–34)NH₂ in COS-7 cells transfected with hP1R-WT (Fig. 4A and Table III). The negligible effects of these substitutions in PTH(1–34) with the intact receptor were also seen in HKRK-B7 and ROS-17/2.8 cells (Tables I and II). However, the [Ala₃,¹⁰,¹²,Arg¹¹,Tyr³⁴]hPTH(1–34)NH₂ analog was 100-fold more potent than [Tyr³⁴]hPTH(1–34)NH₂ in stimulating cAMP with hP1R-delNt (Fig. 4A and B; Table III). Thus, with both the NH₂-terminal PTH fragments and intact PTH (1–34) analogs, the Ala₃,¹⁰,¹² and Arg¹¹ substitutions enhanced signaling potency in the absence of the receptor’s NH₂-terminal extracellular domain. The magnitude of the enhancing effects of these substitutions were similar for the PTH (1–14) fragment and the PTH (1–34) peptide when measured in the context of the truncated receptor. Consistent with this, [Ala₃,¹⁰,¹²,Arg¹¹,Tyr³⁴]hPTH(1–34)NH₂ was 8.6-fold more potent than [Ala₃,¹⁰,¹²,Arg¹¹]rPTH(1–14)NH₂ with hP1R-delNt (Fig. 4B and Table III), a result that suggests a role for the PTH (15–34) domain in interactions to the juxtapamembrane region of the receptor.

**Inhibition by PTH(3–34)**—As a means to address whether the PTH (1–14) analogs bind to the same receptor site as PTH (1–34), we tested the ability of the antagonist [Tyr³⁴]hPTH(3–34)NH₂ to inhibit the signaling responses elicited by both PTH (1–34) and the most potent fragment analog, [Ala₃,¹⁰,¹²,Arg¹¹,Trp¹⁴]rPTH(1–14)NH₂, in ROS 17/2.8 cells. The antagonist, hPTH(3–34), at doses of 0.1 μM and 0.5 μM, caused parallel shifts to the right in the dose-response curves obtained for each agonist ligand without depressing the maximum response attained (Fig. 5). The inhibitory potency of the antagonist was comparable with the two agonists, as for each ~2- and ~10-fold rightward shifts in the responses curves occurred with hPTH(3–34) doses of 0.1 and 0.5 μM, respectively. Thus, PTH (3–34) functions as a simple competitive inhibitor for both PTH (1–34) and the highly modified PTH (1–14) analog.

**Stimulation of Inositol Phosphate Production**—One of the more potent NH₂-terminal peptides in this study ([Ala₃,¹⁰,¹²,Arg¹¹,Trp¹⁴]rPTH(1–14)NH₂) was tested for the ability to stimulate inositol phosphate production in COS-7 cells transfected with hP1R-WT. As shown in Fig. 6, the modified PTH (1–14) analog induced a 3.9-fold increase in total [³²P]inositol phosphate accumulation at a peptide dose of 300 μM; the EC₅₀ of this response was 190 ± 60 μM. The control peptide rPTH(1–34) induced a maximum 4.6-fold increase in inositol phosphate accumulation, and the EC₅₀ of this response was 22 ± 7 nm. No response could be detected for unsubstituted rPTH(1–14).

**DISCUSSION**

In this report we used the rPTH(1–14) peptide sequence to investigate how residues in the NH₂-terminal portion of PTH contribute to function, and to determine whether a fully efficacious NH₂-terminal PTH peptide could be developed in the absence of the PTH (15–34) binding domain. We first extended our previous alanine-scan analysis of the native PTH (1–14) peptide (14) by synthesizing and functionally evaluating 118 different singly substituted PTH (1–14) analogs. The testing of these analogs for cAMP-stimulating potency in stably transfected LLC-PK1 cells (HKRK-B7) confirmed the intolerance of the (1–9) region, as only 7 of 74 substitutions in this region resulted in peptides that retained at least 25% of the activity of native PTH (1–14). It also confirmed the relative tolerance of the (10–14) region, as 41 of 58 of the substitutions here resulted in peptides that were 25% or more as active as native PTH (1–14).

The effects of many of the single substitutions that we analyzed in PTH (1–14) were consistent with the results obtained by others in studies on the same or similar substitutions introduced into PTH (1–34) or related analogs. In general, these other studies confirm the importance of residues in the (1–9) region of PTH (1–34) for biological activity and the relative
Table I

| Peptide                  | EC<sub>50</sub> | E<sub>max(calc)</sub> | E<sub>max(obs)</sub> |
|-------------------------|-----------------|----------------------|---------------------|
| PTH(1–34) analogs       |                 |                      |                     |
| [Nea<sup>21</sup>, Tyr<sup>24</sup>]rPTH(1–34)NH<sub>2</sub> | 0.23 ± 0.05 mM     | 449 ± 43             | 459 ± 23            |
| [Ala<sup>1</sup>, Tyr<sup>24</sup>, Arg<sup>21</sup>]rPTH(1–34)NH<sub>2</sub> | 0.19 ± 0.05 mM     | 442 ± 47             | 422 ± 21            |
| PTH fragment analogs   |                 |                      |                     |
| rPTH(1–14)NH<sub>2</sub> (native) | 5.6 ± 2.5 μM      | 420 ± 25             | 408 ± 20            |
| [Ala<sup>1</sup>, Tyr<sup>24</sup>, Arg<sup>21</sup>]rPTH(1–14)NH<sub>2</sub> | 2.8 ± 0.8 μM       | 375 ± 49             | 437 ± 38            |
| [Ala<sup>1</sup>, Arg<sup>21</sup>]rPTH(1–11)NH<sub>2</sub> | 120 ± 41 μM       | 408 ± 32             | 218 ± 29            |

Fig. 4. cAMP-stimulating activity of PTH analogs with intact and truncated PTH receptors in COS-7 cells.

COS-7 cells transiently transfected with the intact human PTH-1 receptor (hP1R-WT) (left panel) or a truncated hPTH-1 receptor lacking most of the amino-terminal domain (hP1R-delNt) (right panel) were tested for the ability to mediate cAMP accumulation in response to varying doses of [Tyr<sup>34</sup>]hPTH(1–34)NH<sub>2</sub>, [Ala<sup>1</sup>, Tyr<sup>24</sup>, Arg<sup>21</sup>]rPTH(1–34)NH<sub>2</sub>, rPTH(1–14)NH<sub>2</sub>, or [Ala<sup>1</sup>, Tyr<sup>24</sup>, Arg<sup>21</sup>, Trp<sup>14</sup>]rPTH(1–14)NH<sub>2</sub>. The symbols are defined in the figure key. Shown are data (mean ± S.E.) combined from three separate experiments, each performed in duplicate. Single-letter amino acid codes are used.

Table II

| Peptide                  | EC<sub>50</sub> | E<sub>max(calc)</sub> | E<sub>max(obs)</sub> |
|-------------------------|-----------------|----------------------|---------------------|
| hP1R-WT                 |                 |                      |                     |
| [Nea<sup>21</sup>, Tyr<sup>24</sup>]rPTH(1–34)NH<sub>2</sub> | 0.70 ± 0.23 mM     | 226 ± 31             | 220 ± 5             |
| [Tyr<sup>24</sup>]hPTH(1–34)NH<sub>2</sub> | 0.54 ± 0.10 mM     | 226 ± 30             | 218 ± 16            |
| [Ala<sup>1</sup>, Tyr<sup>24</sup>, Arg<sup>21</sup>]rPTH(1–34)NH<sub>2</sub> | 0.33 ± 0.22 mM     | 230 ± 28             | 223 ± 15            |
| rPTH(1–14)NH<sub>2</sub> | 152 ± 35 μM      | 221 ± 22             | 95 ± 7              |
| [Ala<sup>1</sup>, Tyr<sup>24</sup>, Arg<sup>21</sup>]rPTH(1–14)NH<sub>2</sub> | 0.92 ± 0.14 μM     | 224 ± 31             | 224 ± 15            |
| [Ala<sup>1</sup>, Arg<sup>21</sup>]rPTH(1–11)NH<sub>2</sub> | 163 ± 8.2 μM       | 231 ± 26             | 184 ± 16            |
| hP1R-delNt              |                 |                      |                     |
| [Nea<sup>21</sup>, Tyr<sup>24</sup>]rPTH(1–34)NH<sub>2</sub> | 4.7 ± 1.3 μM       | 243 ± 63             | 159 ± 21            |
| [Tyr<sup>24</sup>]hPTH(1–34)NH<sub>2</sub> | 29.1 ± 15.3 μM    | 238 ± 20             | 82 ± 11             |
| [Ala<sup>1</sup>, Tyr<sup>24</sup>, Arg<sup>21</sup>]rPTH(1–34)NH<sub>2</sub> | 0.25 ± 0.16 μM     | 251 ± 39             | 243 ± 17            |
| rPTH(1–14)NH<sub>2</sub> | 313 ± 121 μM     | 222 ± 31             | 56 ± 12             |
| [Ala<sup>1</sup>, Tyr<sup>24</sup>, Arg<sup>21</sup>]rPTH(1–14)NH<sub>2</sub> | 2.4 ± 1.1 μM       | 250 ± 61             | 244 ± 22            |
| [Ala<sup>1</sup>, Arg<sup>21</sup>]rPTH(1–11)NH<sub>2</sub> | 27.0 ± 12.9 μM     | 243 ± 63             | 185 ± 27            |

Table III

| Peptide                  | EC<sub>50</sub> | E<sub>max(calc)</sub> | E<sub>max(obs)</sub> |
|-------------------------|-----------------|----------------------|---------------------|
| hP1R-WT                 |                 |                      |                     |
| [Nea<sup>21</sup>, Tyr<sup>24</sup>]rPTH(1–34)NH<sub>2</sub> | 0.70 ± 0.23 mM     | 226 ± 31             | 220 ± 5             |
| [Tyr<sup>24</sup>]hPTH(1–34)NH<sub>2</sub> | 0.54 ± 0.10 mM     | 226 ± 30             | 218 ± 16            |
| [Ala<sup>1</sup>, Tyr<sup>24</sup>, Arg<sup>21</sup>]rPTH(1–34)NH<sub>2</sub> | 0.33 ± 0.22 mM     | 230 ± 28             | 223 ± 15            |
| rPTH(1–14)NH<sub>2</sub> | 152 ± 35 μM      | 221 ± 22             | 95 ± 7              |
| [Ala<sup>1</sup>, Tyr<sup>24</sup>, Arg<sup>21</sup>]rPTH(1–14)NH<sub>2</sub> | 0.92 ± 0.14 μM     | 224 ± 31             | 224 ± 15            |
| [Ala<sup>1</sup>, Arg<sup>21</sup>]rPTH(1–11)NH<sub>2</sub> | 163 ± 8.2 μM       | 231 ± 26             | 184 ± 16            |
| hP1R-delNt              |                 |                      |                     |
| [Nea<sup>21</sup>, Tyr<sup>24</sup>]rPTH(1–34)NH<sub>2</sub> | 4.7 ± 1.3 μM       | 243 ± 63             | 159 ± 21            |
| [Tyr<sup>24</sup>]hPTH(1–34)NH<sub>2</sub> | 29.1 ± 15.3 μM    | 238 ± 20             | 82 ± 11             |
| [Ala<sup>1</sup>, Tyr<sup>24</sup>, Arg<sup>21</sup>]rPTH(1–34)NH<sub>2</sub> | 0.25 ± 0.16 μM     | 251 ± 39             | 243 ± 17            |
| rPTH(1–14)NH<sub>2</sub> | 313 ± 121 μM     | 222 ± 31             | 56 ± 12             |
| [Ala<sup>1</sup>, Tyr<sup>24</sup>, Arg<sup>21</sup>]rPTH(1–14)NH<sub>2</sub> | 2.4 ± 1.1 μM       | 250 ± 61             | 244 ± 22            |
| [Ala<sup>1</sup>, Arg<sup>21</sup>]rPTH(1–11)NH<sub>2</sub> | 27.0 ± 12.9 μM     | 243 ± 63             | 185 ± 27            |

Tolerance of residues in the (10–14) region (34–38). This correlation between the effects of NH<sub>2</sub>-terminal substitutions on the activities of PTH(1–14) and PTH(1–34) suggests that the shorter peptides interact with a site in the receptor that is also utilized by PTH(1–34). The affinity of these PTH(1–14) analogs for the PTH-1 receptor was still too weak for us to measure by conventional competition binding methods (data not shown). However, in our cAMP inhibition studies directly tested for binding site overlap (Fig. 5), and the results showed that PTH(3–34) induced parallel and quantitatively similar displacements in the activation curves of PTH(1–34) and modified PTH(1–14). These results, together with the observation that a PTH(1–14) analog could activate the phospholipase C-signaling pathway (Fig. 6), further support the hypothesis that the modified PTH(1–14) analogs occupy the same receptor site as that used by PTH(1–34). Our data showing that a PTH(1–14) analog can activate phospholipase C are consistent with the hypothesis that the "activation domain" for this signaling pathway resides at the NH<sub>2</sub> terminus of PTH(1–34) (12).
other bioactive peptides in the data bases (analyzed using the FASTA program of the Genetics Computer Group (Madison WI) software package). Even [Ala<sup>8,10</sup>,Thr<sup>11</sup>]-rPTH(1–14)NH<sub>2</sub> retained closest homology to PTH and PTHrP (73% and 55% identity, respectively). Consistent with this, the signaling responses induced by the PTH fragments were fully dependent on the PTH-1 receptor, as they were inactive in untransfected LLC-PK1 and COS-7 cells (data not shown).

How these substitutions enhance activity is unknown. It is clear that they modify interactions with the juxtamembrane portion of the receptor containing the transmembrane helices and extracellular loops, because they improved potency on the truncated receptor by as much as they did on the intact receptor. The functional intolerance of residues in the (1–9) region, especially Val<sup>8</sup>, Ile<sup>9</sup>, and Met<sup>10</sup>, is at least consistent with the recently reported computer model of the complex formed between PTH(1–34) and the PTH-1 receptor (39), which predicts that these NH<sub>2</sub>-terminal residues of PTH interact with the transmembrane helices and/or extracellular loops of the receptor. Some of the enhancing substitutions may induce a more

**Acknowledgments**—We thank Percy H. Carter and Henry M. Kronenberg for helpful discussion and reading of the manuscript, and Ashok Khatri of the Massachusetts General Hospital Biopolymer Core Facility for peptide synthesis.

REFERENCES

1. Kronenberg, H., Abou-Samra A, Bringhurst, F., Gardella, T., Jüppner, H., and Segre, G. (1997) in Genetics of Endocrine and Metabolic disorders (Thakker, R., ed) pp. 389–420, Chapman & Hall, London.
2. Jüppner, H., Abou-Samra, A-B., Freeman, M., Kong, X-F., Schipani, E., Richards, J., Kolakowski, L. F., Jr., Hock, J., Potts, J. T., Jr., Kronenberg, H. M., and Segre, G. V. (1991) Science 254, 1024–1026.
3. Kolakowski, L. F. (1994) Receptors Channels 2, 1–7.
4. Iida-Klein, A., Guo, J., Xie, L., Jüppner, H., Potts, J. T., Jr., Kronenberg, H. M., Bringhurst, F., Abou-Samra, A-B., and Segre, G. V. (1995) J. Biol. Chem. 270, 6438–6455.
5. Nussbaum, R. S., Rosenblatt, M., and Potts, J. T., Jr. (1980) J. Biol. Chem. 255, 10183–10187.
6. Tregear, G. W., Van Rietschoten, J., Greene, E., Keutmann, H. T., Niall, H. D., Reiv, B., Parsons, J. A., and Potts, J. T., Jr. (1975) J. Endocrinology 93, 1349–1353.
7. Nutt, R. F., Caulfield, M. P., Levy, J. J., Gibbons, S. W., Rosenblatt, M., and McKeen, R. L. (1980) Endocrinology 107, 491–495.
8. Gisslen, A., Pfeutymann, A., Callahan, E., Tregear, G. W., and Potts, J. T., Jr. (1975) J. Biol. Chem. 250, 3199–3203.
9. Carter, P., Jüppner, H., and Gardella, T. (1999) Endocrinology 140, 4972–4981.
10. Dempster, D. W., Cosman, F., Parisien, M., Shen, V., and Lindsay, R. (1995) Endocr. Rev. 16, 690–709; Erratum (1994) Endocr. Rev. 15, 261.
11. Roe, E., Sanchez, S., del Puerto, G., Pierini, E., Bacchetti, P., Cann, C., and McKeen, R. L. (1995) J. Bone Miner. Res. 10, Suppl. 1, S137.
12. Takasu, H., Gardella, T., Luck, M., Potts, J. T., Jr., and Bringhurst, F. (1999) Biochemistry 38, 13453–13460.
13. Neugebauer, W., Barbier, J. R., Sun, W-L., Whitfield, J. F., and Willick, G. E. (1995) Biochemistry 34, 8835–8842.
14. Luck, M., Carter, P., and Gardella, T. (1999) Mol. Endocrinol. 13, 670–680.
15. Bergwitz, C., Jusseaume, S., Luck, M., Jüppner, H., and Gardella, T. (1997) J. Biol. Chem. 272, 28861–28867.
16. Lee, C., Luck, M., Jüppner, H., Potts, J., Kronenberg, H. and Gardella, T. (1995) Mol. Endocrinol. 9, 1299–1278.
17. Bisello, A., Adams, A. E., Sarker, D., Pellegrini, M., Rosenblatt, M., Sara, L., and Chow, M. (1998) J. Biol. Chem. 273, 22498–22505.
18. Gardella, T. J., Jüppner, H., Wilson, A. K., Keutmann, H. T., Bringhurst, F., Abou-Samra, A. B., Segre, G. V., and Potts, J. T., Jr., Nussbaum, S. R., and Kronenberg, H. M. (1994) Endocrinology 135, 1186–1194.
19. Behar, V., Bisello, A., Bitan, B., Rosenblatt, M., and Chow, M. (1999) J. Biol. Chem. 274, 9–17.
20. Bergwitz, C., Gardella, T., Flannery, M., Potts, J. J., Kronenberg, H., and Goldring, S., and Jüppner, H. (1996) J. Biol. Chem. 271, 26469–26472.
21. Stoop, S., Kuestner, B., Serwald, T., Chen, L., and Moore, E. (1995) Biochemistry 34, 1050–1057.
22. Abell, A., Liu, X., and Segaloff, D. (1996) J. Biol. Chem. 271, 4518–4527.
23. Takasu, H., Guo, J., and Bringhurst, F. (1999) J. Bone Miner. Res. 14, 11–20.
24. Majeska, R. J., Rodan, S. B., and Rodan, G. A. (1980) Endocrinology 107, 1494–1503.
25. Yamamoto, I., Shigeno, C., Potts, J. T., Jr., and Segre, G. V. (1988) Endocrinology 122, 1208–1217
26. Shipani, E., Karga, H., Karaplis, A. C., Potts, J. T., Jr., Kronenberg, H. M., Segre, G. V., Abou-Samra, A. B., and Juppner, H. (1993) Endocrinology 132, 2157–2165
27. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
28. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Protein Eng. 10, 1–6
29. Carter, P., Shimizu, M., Luck, M., and Gardella, T. (1999) J. Biol. Chem. 274, 31955–31960
30. Berridge, M., Dawson, R., Downes, C., Hessop, J., and Irvine, R. (1983) Biochem. J. 212, 473–482
31. Bowen, W., and Jerman, J. (1995) Trends Pharmacol. Sci. 16, 413–417
32. Lee, C., Gardella, T., Abou-Samra, A.-B., Nussbaum, S., Segre, G., Potts, J., Kronenberg, H., and Juppner, H. (1994) Endocrinology 135, 1488–1495
33. Mannstadt, M., Luck, M., Gardella, T., and Juppner, H. (1998) J. Biol. Chem. 273, 16890–16896
34. Frelinger, A. L., III, and Zull, J. E. (1984) J. Biol. Chem. 259, 5507–5513
35. Gardella, T. J., Axelrod, D., Rubin, D., Keutmann, H. T., Potts, J. T., Jr., Kronenberg, H. M., and Nussbaum, S. R. (1991) J. Biol. Chem. 266, 13141–13146
36. Gombert, F., Gamse, R., Feyen, J., and Cardinaux, F. (1996) in Peptides: Chemistry, Structure and Biology Proceedings of the 14th American Peptide Symposium June 18–23 (Kaumaya, P., and Hodges, R., eds) pp. 661–662, Mayflower Scientific Limited, Kingswinford, United Kingdom
37. Cohen, F. E., Strewler, G. J., Bradley, M. S., Carlquist, M., Nilsson, M., Ericsson, M., Ciardelli, T. L., and Nissenson, R. A. (1991) J. Biol. Chem. 266, 1997–2004
38. Oldenburg, K. R., Epand, R. F., D’Orfani, A., Vo, K., Selick, H., and Epand, R. M. (1996) J. Biol. Chem. 271, 17582–17591
39. Roiz, C., Pellegrini, M., and Mierke, D. (1999) Biochemistry 38, 6397–6405
40. Barden, J. A., and Kemp, B. E. (1993) Biochemistry 32, 7126–7132
41. Marx, U., Austermann, S., Bayer, P., Adermann, K., Eichert, A., Sticht, H., Walters, S., Schmid, F., Jaenicke, R., Forssmann, W., and Rosch, P. (1995) J. Biol. Chem. 270, 15194–15202
42. Pellegrini, M., Royo, M., Rosenblatt, M., Chorev, M., and Mierke, D. (1998) J. Biol. Chem. 273, 10420–10427
43. Adams, A., Bisello, A., Chorev, M., Rosenblatt, M., and Suva, L. (1998) Mol. Endocrinol. 12, 1673–1683