Mono- and Bicyclic Analogs of Parathyroid Hormone-Related Protein. 1. Synthesis and Biological Studies†

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ABSTRACT: The bioactive conformation of parathyroid hormone-related protein (PTHrP), a single-chain linear peptide structurally similar to parathyroid hormone (PTH), is of considerable interest because PTH and PTHrP both recognize and bind to a shared G-protein-coupled receptor. Both hormones are thought to present a bioactive conformation to the receptor which is substantially α-helical in nature. To better characterize this putative biologically relevant conformation, we prepared a series of conformationally constrained analogs of PTHrP with enhanced α-helical stability. A combination of structural constraint and helix stabilization was achieved through side chain-to-side chain lactam ring formation between Lys^{i} and Asp^{i+4} residues (13-to-17 and 26-to-30) along the PTHrP sequence. Mono- and bicyclic analogs derived from the agonist PTHrP-(1−34)NH_2 and the antagonist PTHrP-(7−34)NH_2 were prepared and characterized in terms of receptor binding and stimulation (or antagonism) of PTH-stimulated adenylyl cyclase activity in osteoblast-like cells. The binding affinity of monocyclic [Lys^{13},Asp^{17}]- (**I**) and bicyclic [Lys^{13},Asp^{17},Lys^{26},Asp^{30}]PTHrP-(1−34)NH_2 (**III**) agonists was in the low nanomolar range and similar to that of the parent linear peptide. Furthermore, their efficacy was in the sub-nanomolar range and about 10-fold higher than that of the corresponding linear parent peptide. Analogs **I** and **III** are the first cyclic PTH/PTHrP receptor agonists and amongst the most potent PTHrP analogs yet designed. The rank-order of potency in the cyclic antagonist series does not correlate with the binding affinities. In light of the positional dependence and the differential effects of lactam bridge formation on the biological activities of agonist vs antagonists, these analogs may provide insight regarding the biologically relevant conformations of PTHrP-derived ligands [Maretto et al. (1997) *Biochemistry 36,* 3300−3307].

Parathyroid hormone-related protein (PTHrP),[1] an alternatively spliced peptide of 139, 141, or 173 amino acids, was originally isolated as a secretory product of tumors associated with the clinical syndrome of humoral hypercalcemia of malignancy. PTHrP is also produced by a variety of normal cells and may act as a paracrine and/or autocrine agent in normal physiology and development (Suva et al., 1987; Martin et al., 1991). It binds with nanomolar affinity to a G-protein-coupled seven-transmembrane-spanning receptor which it shares with parathyroid hormone (PTH), termed the PTH/PTHrP receptor (Jüppner et al., 1991; Jüppner, 1994). The fact that PTH and PTHrP, despite sharing only limited sequence homology, bind to and activate the same receptor suggests that both peptide hormones assume a very similar "bioactive conformation" when bound to the PTH/PTHrP receptor.

The identification of the N-terminal (1−34) portion of both PTH and PTHrP as the sequences which possess full calciotropic activity and the subsequent elucidation of the sychnologic organization (Schwyzer, 1980) of functional domains in the PTH molecule facilitated the design of highly potent antagonists. Truncation of six amino acid residues, the major activation domain ("message domain"), from the N-terminus yields relatively non-homologous 7−34 sequences which were the lead structures for the development of PTH- and PTHrP-derived antagonists with nanomolar potency (Rosenblatt, 1986; Chorev & Rosenblatt, 1994; McKee et al., 1988; Nutt et al., 1990).

Chou-Fasman analysis (Nussbaum et al., 1985; Chorev et al., 1990), strucuture−activity studies and conformational analysis suggested that the propensity for α-helicity correlates with both PTH-like agonist and antagonist activities. Accordingly, a model for the bioactive conformation of ligands suggests the presence of two N- and C-terminal amphiphilic helical domains which are connected by a reversed turn (Choen et al., 1991). This model positions the hydrophobic residues on both helicies inwardly, and the hydrophilic ones

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[1] Abbreviations: Boc, *N-tert*-butyloxycarbonyl; BOP, (benzotryazole-1-yl-oxy)-tris(dimethylamino)phosphonium hexafluorophosphate; CD, circular dichroism; DCC, *N*,*N*′-dicyclohexylcarbodiimide; 2D-NMR, 2-dimentional nuclear magnetic resonance; FAB-MS, fast-atom bombardment mass spectrometry; FBS, fetal bovine serum; Fmoc, *N*-9*H*-fluorenylmethoxycarbonyl; HOBt, *N*-hydroxybenzotriazole; NMP, *N*-methylpyrrolidone; OcHex, cyclohexyl ester; OFm, fluorenylmethyl ester; PBS, phosphate-buffered saline; PTH, parathyroid hormone; [^{125}I]PTH-(1−34)NH_2, [^{125}I][$Nle^{8,18}$,Tyr^{34}]bPTH-(1−34)NH_2; PTHrP, PTH-related protein; PyBOP, (benzotriazole-1-yl-oxy)-tris(pyrrolidino)phosphonium hexafluorophosphate; RP-HPLC, reverse-phase high-performance liquid chromatography; Saos-2/B-10, human osteosarcoma cells.

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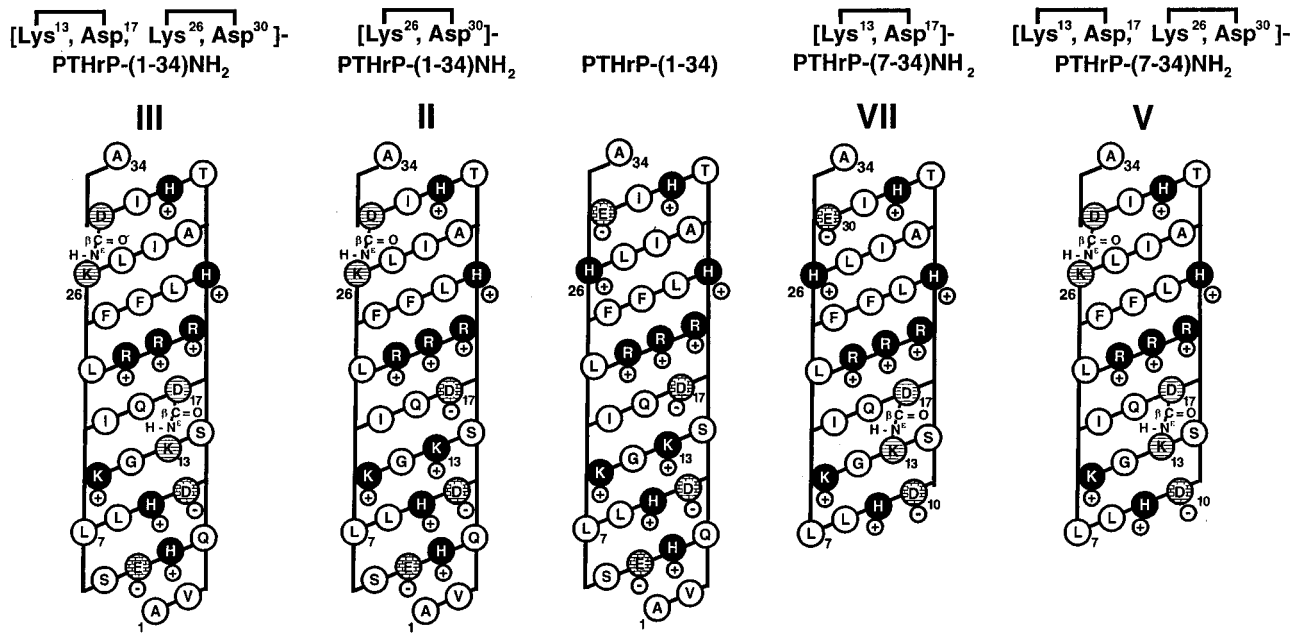

FIGURE 1: Schematic representation of some monocyclic (**II** and **VII**) and bicyclic lactam analogs (**III** and **V**) derived from PTHrP, and of the linear, unmodified PTHrP-(1−34) displayed in an α-helical conformation. Key residues are highlighted as follows: positively charged residues (black), negatively charged residues (checked), and residues participating in the cyclization (striped).

outwardly. Additional conformational studies identify helicity as a prominent conformational feature (Chorev et al., 1995), but do not support the proposed U-shaped folded structure (Klaus et al., 1991; Barden & Kemp, 1991; Strickland et al., 1993).

In an effort to validate the relevance of helicity to PTH-like calciotropic activity, we undertook stepwise reduction of conformational flexibility of PTHrP-(1−34) and PTHrP-(7−34) to stabilize segments of the molecules in an α-helical conformation. Conformational constraint was achieved by side chain-to-side chain ring formation between Lys^{i} and Asp^{i+4} residues in order to enhance α-helical stability. A similar approach was used to enhance α-helical propensity in bioactive peptides, such as growth hormone-releasing factor (GRF) (Felix et al., 1988), corticotropin-releasing factor (CRF) (Miranda et al., 1994), dynorphin A (Lung et al., 1996), neuropeptide Y (NPY) (Bouvier et al., 1992), choecystokinine-8 (CCK-8) (Danho et al., 1991), and calcitonin (Kapurniotu & Taylor, 1995). In general, when bioactivity was associated with α-helicity, introduction of an (*i*)−(*i*+4) side chain-to-side chain lactam bridge resulted in enhanced biological potency. Introduction of a lactam bridge between Lys^{13} and Asp^{17} generated the only reported cyclic PTHrP-based antagonist, [Lys^{13},Asp^{17}]PTHrP-(7−34)NH_2 (**VII**) (Chorev et al., 1991). This analog displays 5−10-fold higher affinity and antagonist activity than the parent linear antagonist PTHrP-(7−34)NH_2. Moreover, circular dichroism (CD) studies in the presence of lipids reveal that the antagonist **VII** possesses a higher degree of helicity than the parent linear peptide (45% vs 30% in the presence of sodium dodecyl sulfate and dimyristoylphosphatidyl glycerol) (Chorev et al., 1993). We concluded that replacement of the potential salt bridge between opposite-charged functions on side chains of Lys^{13} and Asp^{17} with a stable covalent lactam bond contributes to conformational stability and therefore enhances both α-helicity and biological activity.

In this report, we extend the structural restriction, in the form of (*i*)−(*i*+4) side chain-to-side chain lactam bridge formation, toward the C-terminus, namely in positions 26−30, and study the effect of this conformational constraint on both the 1−34 and the 7−34 PTHrP-derived agonist and antagonist series (Figure 1). Some of the resulting mono- and bicyclic PTHrP-(1−34)NH_2-derived ligands are among the most potent PTH/PTHrP receptor agonists reported to date. In the report immediately adjacent to this one (Maretto et al., 1997), we describe detailed conformational analysis of the mono- and bicyclic PTHrP-(7−34) analogs included in this paper. In addition, we assess the relation between the observed biological properties and conformational features such as the propensity, location and nature of the helices, as well as other non-helical conformational motifs.

MATERIALS AND METHODS

Materials. *N*-Boc-Asp(β-OFm), *N*-Boc-Asp(β-OcHex), *N*-Boc-norleucine and *N*-Boc-Lys(ε-Fmoc) were purchased from Bachem (Torrence, CA). All other Boc-protected amino acid derivatives, *p*-methylbenzhydrylamine resin hydrochloride (*p*MBHA·HCl resin), trifluoroacetic acid (TFA), *N*,*N*′-dicyclohexylcarbodiimmide (DCC), *N*-hydroxybenzotryazole (HOBt), diisopropylethylamine (DIEA), and piperidine were obtained from Applied Biosystems (Foster City, CA). (Benzotriazol-1-yl-oxy)-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) was purchased from Richelieu Biotechnologies (St. Hyacinthe, QC, Canada). Benzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate (PyBOP) was purchased from Novabiochem (La Jolla, CA). B&J brand dichloromethane (DCM), *N*-methylpyrrolidone (NMP), *N*,*N*′-dimethylformamide (DMF), and acetonitrile (ACN) were obtained from Baxter (McGraw Park, IL). Hydrogen fluoride (HF) was purchased from Matheson (Secaucus, NJ). *N*,*N*′-Diisopropylcarbodiimide (DIC), anisole, *p*-cresol, *p*-thiocresol, dimethyl sulfide, ethanedithiol, and butanedithiol were obtained from Aldrich

Table 1: Physicochemical Characterization of PTHrP Lactam Analogs **I**−**VI**

	I	**II**	**III**	**IV**	**V**	**VI**
M_w[a]						
calculated	3999	3977	3357	3326	3307	3440
found	4000	3977	3357	3324	3306	3438
HPLC						
t_r (min)[b]	18.05	18.77	19.62	18.85	19.87	23.76
k' [c]	7.47	7.81	8.21	7.86	8.28	10.11
amino acid analysis[d]						
Asx	2.09 (2)	3.12 (3)	2.98 (3)	2.97 (3)	2.87 (3)	2.93 (3)
Thr	0.68 (1)	1.06 (1)	0.74 (1)	1.01 (1)	1.07 (1)	0.94 (1)
Ser	1.00 (2)	1.62 (2)	1.01 (2)	0.87 (1)	0.85 (1)	0.85 (1)
Glx	4.66 (5)	3.16 (3)	3.37 (3)	1.17 (1)	1.08 (1)	1.13 (1)
Gly	1.10 (1)	1.06 (1)	1.05 (1)	0.99 (1)	0.97 (1)	0 (0)
Ala	3.18 (3)	2.86 (3)	3.29 (3)	1.99 (2)	2.23 (2)	1.97 (2)
Val	0.99 (1)	0.82 (1)	1.01 (1)	0 (0)	0 (0)	0 (0)
Ile	2.66 (3)	3.05 (3)	2.77 (3)	2.94 (3)	3.02 (3)	2.88 (3)
Leu	5.26 (5)	5.06 (5)	5.26 (5)	5.04 (5)	5.13 (5)	6.06 (6)
Phe	2.13 (2)	2.15 (2)	2.20 (2)	2.10 (2)	2.09 (2)	1.97 (2)
His	5.10 (5)	3.85 (4)	4.16 (4)	2.90 (3)	2.99 (3)	3.02 (3)
Lys	2.04 (2)	3.08 (3)	3.01 (3)	2.99 (3)	2.72 (3)	2.09 (2)
Arg	3.11 (3)	3.06 (3)	3.12 (3)	2.99 (3)	2.72 (3)	2.09 (2)

[a] Molecular weights were measured by FAB−MS, positive mode with thioglycerol matrix. [b] Reverse-phase HPLC analyses were carried out on a Vydac C-18, 300 Å column (4.6 × 150 mm, 5 μm) at a flow rate of 1.0 mL/min and monitored at 220 nm. Linear gradient from 20% to 50% (v/v) eluant B in A over 30 min, where eluant A is 0.1% (v/v) TFA in water and eluant B is 0.1% (v/v) TFA in acetonitrile. [c] $k' = (t_r - t_0)/t_0$, where t_r and t_0 are the retention times of the peptide and the front, respectively, in the conditions described in *b*. [d] Peptides were hydrolyzed on a Pico-Tag instrument and analyzed on an Applied Biosystems Amino Acid Analyzer. Due to hydrolysis conditions, Ser is partially degraded and Trp is not detectable.

(Milwaukee, WI). Na[^{125}I] was obtained from Amersham Corp. (Arlington Heights, IL). IodoGen was purchased from Pierce Chemical Co. (Rockford, IL). RPMI 1640, fetal bovine serum (FBS), trypsin, and PBS were obtained from Gibco-BRL (Gaithersburg, MD). Adenosine and 3-isobutyl-1-methylxanthine (IBMX) was purchased from Research Biochemical (Natick, MA). Tissue culture disposable and plasticware were obtained from Corning (Corning, NY). All other reagents were purchased from Sigma (St. Louis, MO). Radioiodination of [Nle^{8,18},Tyr^{34}]bPTH-(1−34)NH_2 was carried out as previously described (Roubini et al., 1992).

Peptide Synthesis and Purification. The peptides were synthetized on an Applied Biosystems 430A peptide synthesizer, using Nα-Boc and side-chain-benzyl protecting groups and DCC-HOBt-based chemistry and protocols detailed in version 1.40 of HOBt/NMP cycles software from Applied Biosystems. The overall synthesis and formation of the lactam(s) on the resin was performed as described (Chorev et al., 1991). In the synthesis of peptides **I** and **III**, cyclization with BOP was followed by recoupling in the presence of PyBOP. The peptides were cleaved from the resin and fully deprotected by liquid HF in the presence of 10% anisole except for peptide **VI** (containing Trp) which was deprotected and cleaved by the "low/high" procedure (Tam et al., 1983). Peptides were purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) as described (Chorev et al., 1990). Purity of the peptides was assessed by analytical RP-HPLC employing a Vydac C18 300 Å (4.6 × 150 mm, 5 μm) column at a flow rate of 1.0 mL/min, using a variety of linear gradient profiles. k' values (derived from the same gradient profile for all the peptides) are shown in Table 1, structural integrity was confirmed by FAB-MS and amino acid analysis (see Table 1).

Cell Culture. Human osteosarcoma Saos-2/B-10 cells (Rodan et al., 1989) were cultured in RPMI 1640 media supplemented with 2 mM glutamine and 10% FBS. Cells were subcultured weekly as described (Nakamoto et al., 1995).

PTH/PTHrP Receptor Binding Assays. Binding assays were performed in Saos-2/B-10 cells as described (Nakamoto et al., 1995) using HPLC-purified [^{125}I][Nle^{8,18},Tyr^{34}]bPTH-(1−34)NH_2 as radioligand (Goldman et al., 1988). Results are expressed as percentage of specific binding; values represent the mean ± SEM of triplicates from three individual experiments.

Adenylyl Cyclase Assays. Adenylyl cyclase assays were performed in Saos-2/B-10 cells as described (Nakamoto et al., 1995). Results are expressed as percentage of maximal stimulation obtained in the presence of 10^{-6} M PTH-(1−34). Inhibition experiments were performed using 5×10^{-9} M PTH-(1−34), which also represents maximal stimulation, in the presence of PTHrP antagonists at a concentration range of 10^{-9} to 10^{-6} M. Values represent the mean ± SEM of triplicates from three individual experiments.

Data Analysis. Binding constants (K_b), activation constants (K_m), and inhibitory constants (K_i) for PTH-stimulated adenylyl cyclase were calculated according to Cheng and Prusoff (1973).

RESULTS

Physicochemical Characterization of the Peptides. Table 1 summarizes the physicochemical properties of the PTHrP lactam analogs. The purity of analogs **I**−**VI** exceeded 98%. Amino acid analysis and molecular weights (determined by FAB-MS) of the peptides were in full agreement with the calculated values (Table 1). As anticipated, k' values, used as a parameter of overall hydrophobicity, were higher for the bicyclic analogs **III** and **V** than for the corresponding monocyclic analogs **I**, **II** and **IV**.

Biological Activities of the Peptides. Receptor affinity and efficacy in stimulating (for agonists **I**−**III**) and inhibiting (for antagonists **IV**−**VII**) PTH-stimulated adenylyl cyclase

Table 2: Biological Activities of the PTHrP Lactam Analogs[a] (in nM)

	K_b[b]	K_m[c]	K_i[d]
PTHrP-(1−34)NH_2[e]	1.0 ± 0.05	0.57 ± 0.04	
I [Lys^{13},Asp^{17}]	3.2 ± 0.8	0.17 ± 0.03	
II [Lys^{26},Asp^{30}]	410 ± 180	19 ± 1	
III [Lys^{13},Asp^{17},Lys^{26},Asp^{30}]	2.1 ± 0.6	0.22 ± 0.02	
PTHrP-(7−34)NH_2	170 ± 8.5		140 ± 20
IV [Lys^{26},Asp^{30}]	31 ± 8	>1000[f]	>1000[g]
V [Lys^{13},Asp^{17},Lys^{26},Asp^{30}]	95 ± 5		130 ± 34
VI [Leu^{11},D-Trp^{12},Lys^{26},Asp^{30}]	650 ± 49		65 ± 14
VII [Lys^{13},Asp^{17}]	20 ± 2.4		88 ± 5

[a] The values in the table are mean ± SEM of three independent experiments performed in triplicate. [b] Competitive inhibition of binding of [^{125}I]PTH-(1−34) in Saos-2/B-10 cells. [c] Agonist-stimulated adenylyl cyclase activity in Saos-2/B-10 cells. [d] Inhibition of PTH-(1−34)-stimulated adenylyl cyclase (5×10^{-9} M) in Saos-2/B-10 cells. [e] The values for the standard reference compound PTH-(1−34) are $K_b = 1.40 \pm 0.07$ nM and $K_m = 1.70 \pm 0.08$ nM. [f] At 1000 nM, stimulation of adenylyl cyclase was less than 15%. [g] At 1000 nM, it inhibits less then 50% of PTH-(1−34)-stimulated adenylyl cyclase activity.

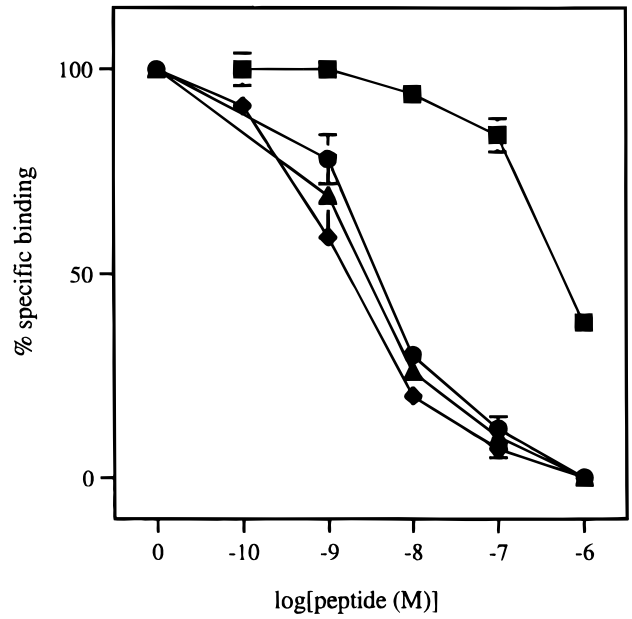

FIGURE 2: Radioreceptor binding curves of the lactam PTHrP-(1−34) analogs in Saos-2/B-10 cells. PTH-(1−34) (◆); analog **I** (●); analog **II** (■); analog **III** (▲). Curves represent a single binding experiment carried out in triplicates. Similar results were obtained in two additional independent experiments.

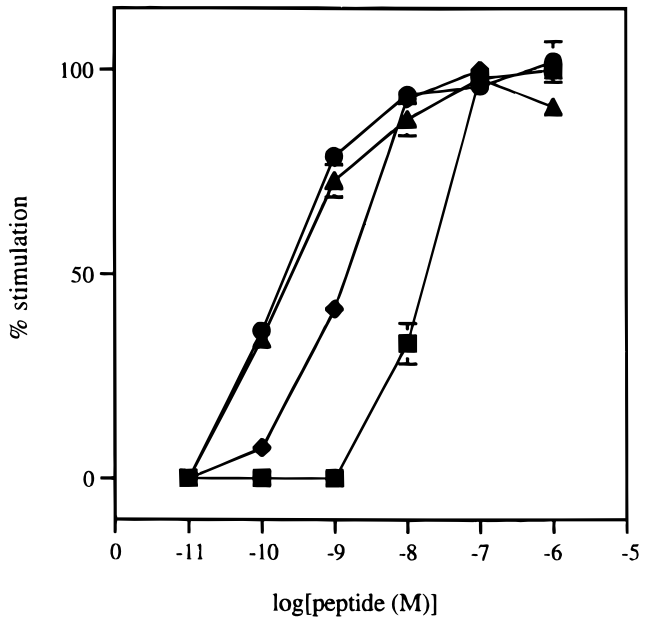

FIGURE 3: Dose−response curves for the stimulation of adenylyl cyclase by lactam PTHrP-(1−34) analogs in Saos-2/B-10 cells. PTH-(1−34) (◆); analog **I** (●); analog **II** (■); analog **III** (▲). Curves represent a single adenylyl cyclase experiment carried out in triplicates. Similar results were obtained in two additional independent experiments.

were determined in human osteosarcoma Saos-2/B-10 cells expressing the PTH/PTHrP receptor (Rodan et al., 1989). The results are displayed in Figures 2−5 and summarized in Table 2.

Agonists. Competition for binding of [^{125}I]PTH-(1−34)NH_2 and dose-response curves for the series of mono- and bicyclic agonists **I**−**III** are displayed in Figures 2 and 3, respectively. The competitive inhibition of binding and the agonist-stimulated adenylyl cyclase activity are summarized in Table 2.

Midregion lactam-ring formation either in the mono- or bicyclic analogs, **I** and **III**, respectively, has a distinct potentiation effect on both stimulation of adenylyl cyclase and receptor binding compared to the linear parent agonist PTHrP-(1−34)NH_2. Both the monocyclic analog **I** and the bicyclic analog **III** display low nanomolar affinity (K_b = 3.2 and 2.1 nM, respectively) and sub-nanomolar efficacy (K_m = 0.17 and 0.22 nM, respectively). The C-terminal monocyclic lactam **II** has ∼100-fold lower efficacy and ∼150-fold lower affinity than the midregion lactam containing analogs (**I** and **III**). It appears that the impact of a midregion lactam ring (Lys^{13}-to-Asp^{17}) has a dominant agonist-like potentiating effect which cannot be diminished by the presence of an additional C-terminal lactam ring (Lys^{26}-to-Asp^{30}).

Antagonists. The competition binding curves for the PTHrP-(7−34)-derived cyclic lactams (**IV**−**VI**) and the previously reported antagonist **VII** (Chorev et al., 1991) are shown in Figure 4 and summarized in Table 2. The monocyclic antagonists **IV** and **VII** display similar binding affinities for the hPTH/PTHrP receptor present in Saos-2/B-10 cells (K_b = 31 and 20 nM, respectively). In this radioreceptor assay, both monocyclic antagonists **IV** and **VII**

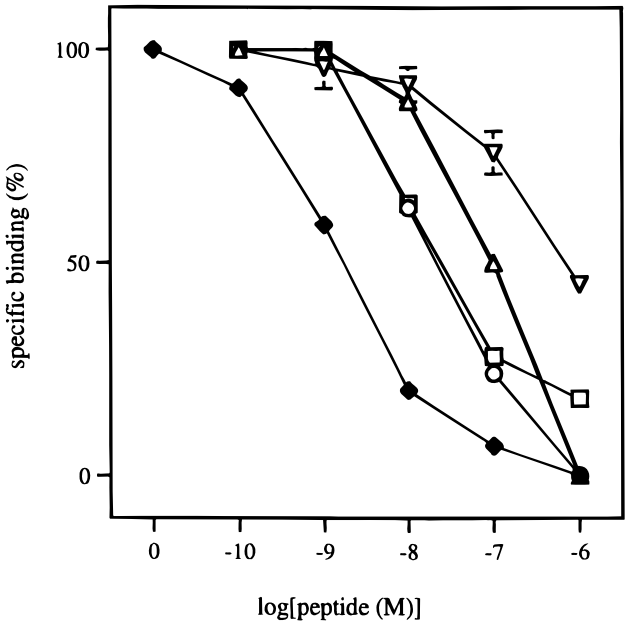

FIGURE 4: Binding competition curves of the lactam PTHrP-(7−34) analogs in Saos-2/B-10 cells. PTH-(1−34) (◆); analog **IV** (□); analog **V** (△); analog **VI** (▽); analog **VII** (○). Curves represent a single binding experiment carried out in triplicates. Similar results were obtained in two additional independent experiments.

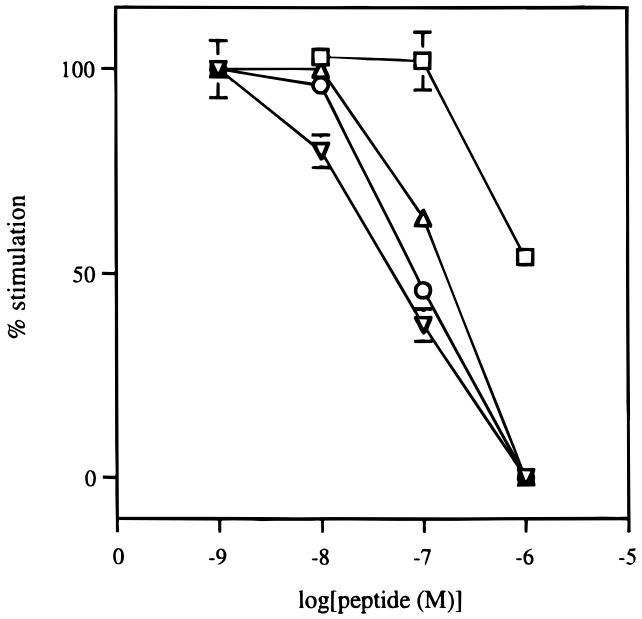

FIGURE 5: Dose−response curves for the inhibition of 5 nM [Nle^{8,18}, Tyr^{34}]bPTH-(1−34)NH_2-stimulated adenylyl cyclase for the PTHrP-(7−34) analogs in Saos-2/B-10 cells. Analog **IV** (□); analog **V** (△); analog **VI** (▽); analog **VII** (○). Curves represent a single inhibition of PTH-stimulated adenylyl cyclase experiment carried out in triplicates. Similar results were obtained in two additional independent experiments.

display about 8-fold higher affinity than the parent linear antagonist PTHrP-(7−34)NH_2. The substitution of His^{26} with Lys and Glu^{30} with Asp combined with lactam bridge formation between the side chains is well-tolerated, as evident from the high affinity of the C-terminal lactam-containing antagonist **IV**. Interestingly, the bicyclic antagonist **V**, which combines the individual cyclizations included in the monocyclic analogs **VII** and **IV**, has about 4-fold lower affinity than either of the monocyclic analogs. No additivity is observed upon combination of the midregion and C-terminal cyclizations into a single analog.

Previously, concomitant substitution of Lys^{11} and Gly^{12} in the linear antagonist PTHrP-(7−34)NH_2 was found to remove partial agonism and potentiate, by about 110-fold, antagonist-like activity (Nutt et al., 1990; McKee et al., 1990). Combination of two distinct antagonist-enhancing structural modifications, namely substitution with Leu^{11}-D-Trp^{12} and Lys^{26}-to-Asp^{30} lactamization as in **VI**, did not result in an additive effect on agonist potency. The Leu^{11}-D-Trp^{12}-substituted monocyclic antagonist **VI** had lower affinity (K_b = 650 nM) than either the linear [Leu^{11},D-Trp^{12}]PTHrP-(7−34)NH_2 (K_b = 27.7 nM; Roubini et al., 1992) or the corresponding monocyclic antagonist **IV** (K_b = 31 nM). This result is reminiscent of a previous attempt to combine the similar activity enhancing modifications, Leu^{11}-D-Trp^{12} and Lys^{13}-to-Asp^{17} lactamization, in a single analog (Chorev et al., 1993). The antagonist [Leu^{11},D-Trp^{12},Lys^{13},Asp^{17}]PTHrP-(7−34)NH_2 was less potent than either the modified linear or the monocyclic lactam (**VII**) antagonists.

The rank-order of antagonist activities (K_i) did not always correspond to the above-listed receptor affinities (Figure 5, Table 2). A major discrepancy is noted for monocyclic antagonist **IV** (K_b = 31 nM and at 1 μM the level of inhibition observed is less than 50%). Analog **IV** is at the same time a weak agonist: at a concentration of 1 μM, it stimulates adenylyl cyclase to less then 15% of the maximal response observed in the presence of the same concentration of our reference agonist [Nle^{8,18},Tyr^{34}]bPTH-(1−34)NH_2.

DISCUSSION

Rational design of highly potent PTH/PTHrP receptor ligands requires understanding of the details of agonist and antagonist bioactive conformations. One approach to overcome the inherent flexibility presented in long linear single-chain peptides, which are able to assume numerous co-existing, energetically similar and dynamically equilibrating conformations, is to introduce structural constraints which will reduce conformational freedom (Hruby, 1982; Kessler, 1982; Veber & Freidinger, 1985; Milner-White, 1989). Provided bioactivity is maintained when conformation is stabilized, we assume that the more structurally constrained the analog, the more the constrained conformation approaches the bioactive one and the better the chances to identify conformational motifs relevant to the biologically active conformation. Conformational analysis of bioactive, structurally constrained ligands of the PTH/PTHrP receptor may provide important insight regarding the receptor-bound conformation assumed by both hormones. This approach is particularly appealing considering the difficulties inherent in attempting to isolate hormone−receptor complexes for a seven transmembrane G-protein-coupled receptor and elucidating their conformation.

This work extends our initial observation (Chorev et al., 1991) in which structural constraint in the form of a Lys^{13}-to-Asp^{17} lactam bridge between side chains of residues *i* and *i*+4 in PTHrP-(7−34)NH_2 (analog **VII**) enhances both the antagonist potency as well as the propensity for α-helix formation. Interestingly, a lactam bridge in an identical location, but in the parent agonist PTHrP-(1−34)NH_2, generates an agonist, [Lys^{13},Asp^{17}]PTHrP-(1−34)NH_2 (**I**), of

enhanced potency. This suggests that α-helicity in the midregion of PTHrP is a common bioactive conformational motif for both PTHrP-derived agonists and antagonists.

The (*i*)−(*i*+4) lactamization between Lys and Asp, substituting His^{26} and Glu^{30}, respectively, in PTHrP-(1−34) generates agonist **II** which possesses modest receptor affinity and efficacy (Table 2).

The same modification in the antagonist PTHrP-(7−34)NH_2 produces analog **IV** which displays a non-classical pharmacological profile (Table 2). The moderate receptor affinity was associated with both very weak PTH-like agonist and antagonist activities. We do not anticipate from analog **IV**, an N-terminal truncated analog missing the most crucial activation domain, to be a potent PTH/PTHr receptor agonist. Apparently, it behaves like a very weak partial agonist (see Table 2). The mechanism by which these partial agonists interact with the receptor is quite obscure. Therefore, currently we can not explain this lack of correlation between binding affinity and biological efficacy.

As previously observed for antagonist **VII** (Chorev et al., 1993), the combination of hydrophobic substitutions, Leu^{11}-D-Trp^{12}, with conformational constraint in the form of lactam Lys^{i},Asp^{i+4}, as in analog **VI**, proved to be non-additive in terms of activity enhancement. However, circular dichroism (CD) and 2-D NMR analysis, followed by restrained distance geometry and molecular dynamic studies of the highly potent antagonist [Leu^{11},D-Trp^{12}]PTHrP-(7−34)NH_2 in 50% 2,2,2-trifluoroethanol (TFE) reveal an extended α-helical segment spanning residues 16−32 (Chorev et al., 1994). We anticipate that the incorporation of an (*i*)−(*i*+4) side chain lactam bridge in this region may further stabilize the α-helical conformation. Therefore, it is not clear to us why antagonist **VI** is much less potent than the corresponding linear antagonist [Leu^{11},D-Trp^{12}]PTHrP-(7−34)NH_2 and without further details regarding the structure of PTH/PTHrP antagonist−receptor complexes, we cannot explain these observations.

A similar modification, [Lys^{26},Asp^{30}]hPTH-(20−34), was reported to enhance α-helical propensity and to stimulate membrane-bound protein kinase C activity in osteoblastic cells (Neugebauer et al., 1994). However, it is not clear if this effect is PTH/PTHrP receptor-mediated.

The most structurally constrained PTHrP analogs generated to date are the bicyclic lactams **III** and **V**. Each of them contains a 20-membered ring in the midregion and another toward the C-terminus. The addition of a second lactam ring in the C-terminal region to the most potent monocyclic antagonist, which contains a midregion lactam ring (**VII**), generates a moderately potent antagonist **V** (Table 2). This remarkable level of structural constraint is well-tolerated, but does not result in either exceptional affinity or potency for inhibiting PTH-stimulated adenylyl cyclase. The addition of a second lactam ring may either destabilize an antagonist-related bioactive conformation or disrupt an important antagonist−receptor interaction. Due to the non-classical pharmacological profile of the monocyclic analog [Lys^{26},Asp^{30}]PTHrP-(7−34)NH_2, further analysis of structure−activity in the cyclic antagonist series (**IV**, **V** and **VII**) must wait for detailed conformational analysis.

The bicyclic agonist **III**, which contains two lactam bridges (between residues in position 13-to-17 and 26-to-30), has a similar pharmacological profile to the monocyclic agonist **I**, which has a single lactam bridge formed between residues in positions 13 and 17. The addition of a second lactam bridge at Lys^{26}-to-Asp^{30} seems to be a silent modification which neither enhances nor disrupts the favorable effects of the Lys^{13}-to-Asp^{17} lactam ring formation. In this respect, the combination of the midregion and the C-terminal lactam bridges in the 1−34 sequence is better tolerated than the same combination in the 7−34 antagonist sequence. Comparison of the biological profiles of monocyclic agonists **I** and **II** suggests that the stabilizing effect of a midregion lactam on α-helicity is much more beneficial to bioactivity than the effect of the C-terminal lactam ring. It suggests the possibility of a temporal process in which stabilization of helicity at the N-terminal portion of the PTHrP agonist must precede formation of the same conformational motif at the C-terminal region. Alternatively, it is possible that a sufficiently high population of the optimal agonist-related bioactive conformational species is already achieved in the monocyclic Lys^{13}-to-Asp^{17} lactam-containing analog, and that the addition of the second lactam bridge at the C-terminus merely stabilizes it further. Currently, the highly constrained agonist of enhanced activity, [Lys^{13},Asp^{17},Lys^{26},Asp^{30}]PTHrP-(1−34)NH_2 (**III**), is the best candidate for further and detailed conformational analysis since it is plausible that its predominant conformation in solution will correspond to the agonist bioactive conformation.

The current series of mono- and bicyclic lactams derived from PTHrP reveals some interesting patterns of structure−activity relations. Midregion lactam rings in both PTHrP-derived sequences, 1−34 (analog **I**) and 7−34 (analog **VII**), yield some of the most active agonists and antagonists. Introduction of a C-terminal lactam ring in the PTHrP-derived sequences 1−34 (analog **II**) and 7−34 (analog **IV**) is accompanied by loss of potency compared to the corresponding linear peptides. The combination of midregion and C-terminal lactams in a single structure (analogs **III** and **V**) has different consequences when introduced into the 1−34 compared to the 7−34 sequence. Addition of the C-terminal lactam bridge to a midregion lactam bridge in the 1−34 sequence (analog **III**) appears to be neutral in its effect on affinity and efficacy (cf. analogs **I** and **III**). In contrast, addition of the C-terminal lactam bridge to a midregion lactam bridge in the 7−34 sequence (analog **V**) results in a significant loss of both affinity and activity (cf. **VII** and **V**). This suggests that helicity as a conformational motif plays different roles in the bioactivity of agonists vs antagonists. Future detailed conformational analysis may identify mechanism(s) responsible for these differences.

In conclusion, we synthesized and evaluated biologically a series of conformationally constrained analogs of PTHrP which includes both agonists and antagonists. Two analogs, monocyclic **I** and bicyclic **III**, were identified as highly potent agonists, displaying 2−3-fold higher efficacy than the linear parent, PTHrP-(1−34)NH_2. Thus, analogs **I** and **III** are the first cyclic agonists and amongst the most potent PTHrP-derived ligands designed to date. As reported in the accompanying paper, the effect of (*i*)−(*i*+4) lactamization on the predominant secondary structure is dependent on the position of lactam ring formation (Maretto et al., 1997). We anticipate that carrying out structure-conformation−

activity studies in an iterative manner on smaller, constrained, peptidic or partially non-peptidic compounds will lead to design of advantageous analogs for therapeutic utility.

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