Receptor selectivity from minimal backbone modification of a polypeptide agonist

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Human parathyroid hormone (PTH) and N-terminal fragments thereof activate two receptors, hPTHR1 and hPTHR2, which share ∼51% sequence similarity. A peptide comprising the first 34 residues of PTH is fully active at both receptors and is used to treat osteoporosis. We have used this system to explore the hypothesis that backbone modification of a promiscuous peptidic agonist can provide novel receptor-selective agonists. We tested this hypothesis by preparing a set of variants of PTH(1–34)-NH_2 that contained a single β-amino-acid residue replacement at each of the first eight positions. These homologs, each containing one additional backbone methylene unit relative to PTH(1–34)-NH_2 itself, displayed a wide range of potencies in cell-based assays for PTHR1 or PTHR2 activation. The β-scan series allowed us to identify two homologs, each containing two α→β replacements, that were highly selective, one for PTHR1 and the other for PTHR2. These findings suggest that backbone modification of peptides may provide a general strategy for achieving activation selectivity among polypeptide-modulated receptors, and that success requires consideration of both β^2- and β^3-residues, which differ in terms of side-chain location.

α/β-peptides | backbone modification | GPCR signaling | subtype-selective agonists | molecular recognition

G-protein-coupled receptors (GPCRs) in class B are modulated by long polypeptide hormones (≥25 residues) and control a wide range of physiological functions (1). Many natural agonists of class B GPCRs can activate more than one receptor, which is consistent with the fact that orthosteric sites of GPCR subtypes are often highly conserved (2). For example, human parathyroid hormone (hPTH) activates two receptors, hPTHR1 and hPTHR2, which share ∼51% sequence similarity (3). hPTHR1 is known to play a key role in regulating calcium homeostasis and tissue development, but the biological function of hPTHR2 remains to be fully established. hPTHR2 may be involved in nociception and spermatogenesis (4, 5). In addition to the dual-specific agonist PTH, each receptor has a natural agonist that is selective, parathyroid hormone related protein (PTHrP) for activation of PTHR1, and TIP39 for PTHR2 (6, 7). The N-terminal fragments PTH(1–34) and PTHrP(1–36) manifest full agonist activity, and the sequence alignment (Fig. 1*A*) shows that there is significant similarity between these two polypeptides, particularly near the N termini. Replacing Ile-5 of PTH(1–34) with His, as found in PTHrP, generates a PTHR1-selective agonist (8, 9). Vasoactive intestinal peptide (VIP) offers another example of hormone polyspecificity: VIP activates the receptors VPAC1 and VPAC2 (10). In this case, no natural receptor-selective agonist is known, and identification of VIP analog peptides that are selective for VPAC1 or VPAC2 required evaluation of many synthetic variants (11, 12).

Approaches to enhance polypeptide agonist specificity have to date been largely focused on side-chain variation, either via evolution or chemical modification, although more exotic modifications have been examined as well (13–15). In the studies reported here, we have asked whether modification of the peptide backbone without changing the side chains, a strategy that is complementary to side-chain alteration, could lead to selectivity in receptor activation. We implemented backbone modification by replacing natural α-amino-acid residues with homologous β-amino-acid residues (Fig. 1*B*). A few prior reports have described the impact of α→β replacement on the receptor-binding selectivities of short peptides (16–19). For example, replacing a single natural residue with a cyclic β-residue in a 12-mer peptide corresponding to the C terminus of neuropeptide Y (NPY) altered binding preferences among NPY receptors (16), and single α→β replacements in the 8-mer angiotensin II strongly reduced affinity for one of the natural receptors but caused only moderate binding reduction for the other natural receptor (18). Our studies are distinct from these precedents in that we focus on a longer peptide agonist, corresponding to the first 34 residues of human parathyroid hormone (PTH). This fragment is the active agent in teriparatide, which is used to treat osteoporosis (20, 21). The size of this peptide offers the prospect of varying agonist properties independently of binding.

The C-terminal portion of PTH(1–34) forms an α-helix and engages the extracellular domain of PTHR1 (22); this interaction is believed to provide a major contribution to the hormone's affinity for the receptor (23, 24). The N-terminal portion of PTH interacts with the transmembrane domain of the receptor, affecting receptor conformation in a way that is sensed by G proteins and other cytosolic proteins to initiate intracellular signaling (25, 26). Our experimental design emerged from the hypothesis that backbone modifications near the N terminus of PTH(1–34)-NH_2 might alter receptor activation behavior

Significance

G-protein-coupled receptors (GPCRs) mediate diverse physiological processes and are targets of many therapeutic agents. GPCR subfamilies comprise related receptors that can be activated by the same or similar agonists but manifest distinct functions. Subtype-selective agonists are valuable as tools for fundamental research and as drug candidates with minimal side effects. It is challenging, however, to develop agonists with differentiated activities at structurally related receptors. We started from a human parathyroid hormone fragment that is used to treat osteoporosis, and developed subtype-selective variants by modifying the peptide backbone while maintaining the natural side chains. Our findings suggest that backbone modification, which has received little prior attention in terms of tailoring polypeptide hormone specificity, may represent a general source of receptor-selective agonists.

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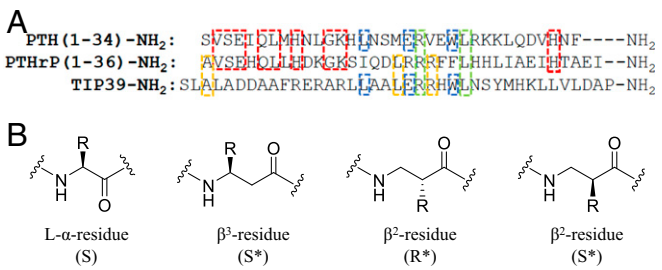


Fig. 1. Sequence of several PTHR agonists and molecular structures of homologous β-amino acids. (*A*) Sequence comparison of PTH(1–34)-NH_2, PTHrP(1–36)-NH_2, and TIP39-NH_2. Red, identical residues between PTH and PTHrP; yellow, identical residues between PTHrP and TIP39; blue, identical residues between PTH and TIP39; green, identical residues shared by all of the three peptides. (*B*) Generic structures of β-amino-acid residues employed in this work; a generic L-α residue is shown for comparison. Most L-α residues have *S* absolute stereochemistry. β^3-Homoamino acids stereospecifically prepared from proteinogenic L-α amino acids are designated as *S** configuration, based on the absolute configuration for (*S*)-β^3-hAla. The designations *S** and *R** are applied to β^2-residues as shown, based on the absolute configurations of the two β^2-hAla enantiomers.

without dramatically influencing affinity. We tested the design hypothesis by asking whether α→β replacement could generate analogs of PTH(1–34)-NH_2 that display selective activation of either PTHR1 or PTHR2 while retaining the natural complement of side chains. We anticipated that an α→β replacement near the N terminus would cause subtle changes, relative to PTH itself, in the 3D presentation of agonist side chains to the receptor. To explore as many variations in side-chain arrangement as possible, we evaluated two types of β-homologs, β^2 (side chain adjacent to carbonyl) and β^3 (side chain adjacent to nitrogen), in replacements at each of the first eight residues of PTH(1–34)-NH_2.

The studies presented below led to discovery of two receptor-selective analogs, each containing two α→β replacements near the N terminus of PTH(1–34)-NH_2. Inclusion of β^2-residues proved to be essential for achieving agonist selectivity. These findings raise the possibility that backbone modification may be broadly useful in generating hormone analogs with tailored activities. This prospect is important because of the growing clinical significance of peptide hormones and their analogs for treatment of human disease (27–29).

Results and Discussion

"β-Scan" of the N-Terminal Portion of PTH(1–34)-NH_2. Many enantiopure β^3-homoamino acids with protecting groups necessary for solid-phase peptide synthesis are commercially available, but only a few protected β^2-homoamino acids can be purchased. This practical distinction has skewed the functional evaluation of peptides containing β-amino-acid residues toward β^3-residues. Most prior work on peptides that contain α→β replacements (α/β-peptides) has focused on β^3-residues that maintain the configuration of L-α-amino acids (18, 19, 30–35), which means *S* for most β^3-residues but *R* in a few cases, such as β^3-hSer or β^3-hThr. Residues with this absolute stereochemistry, which we designate *S** here [in other words, (*R*)-β^3-hSer is designated (*S**)-β^3-hSer] can participate in right-handed α-helix–like secondary structures, as demonstrated crystallographically for numerous α/β-peptides containing 25–33% β-residues distributed among L-α residues (36–38). In contrast, much less is known about the conformational or biological properties of α/β-peptides containing β^2-residues (39–43). In our N-terminal β-scan of PTH(1–34)-NH_2, each of the first eight residues was replaced by the (*S**)-β^3, the (*S**)-β^2, or the (*R**)-β^2 homolog [the designations *S** and *R** indicate that absolute configuration corresponds, respectively, to that of (*S*)- or (*R*)-β^2- or β^3-homoalanine]. For Met-8, the two β^2-homonorleucine (β^2-hNle) enantiomers were employed.

The effects of single α→β replacements on PTHR1 and PTHR2 agonist activity are evaluated in HEK-293 cells that have been engineered to express the appropriate receptor (Fig. 2 and *SI Appendix*, Fig. S1 *A–C*). GloSensor-based detection of cAMP provides a readout of receptor activation (44). Consistent with previous reports (45), PTH(1–34)-NH_2 is very active in both assays (EC_{50} = 0.38 nM for PTHR1, and EC_{50} = 0.87 nM for PTHR2).

For PTHR1 activation, different patterns of substitution tolerance were observed among the α→β^2 and α→β^3 replacements. All three isomeric β-residues were well tolerated in place of Ser-1 or Leu-7, and none of the three was tolerated in place of Ser-3 or Glu-4. At the remaining positions, variable responses to α→β replacement were observed. Thus, for Val-2, the β^3-replacement has little effect on agonist potency, but both β^2-replacements cause significant declines in potency. For Ile-5, the β^3- and (*R**)-β^2 replacements cause modest activity declines, but the (*S**)-β^2 replacement matches PTH(1–34)-NH_2 in activity. Use of (*S**)-β^2-hGln at position 6 has no effect on agonist activity, but placing either β^3-hGln or (*R**)-β^2-hGln at this site causes a substantial activity decline. Both enantiomers of β^2-hNle are well tolerated in place of Met-8, but use of either β^3-hMet or β^3-hNle at this position causes a substantial decline in activity. The overall trend among α→β^3 replacements is consistent with a previously reported β^3-scan of PTH(1–34)-NH_2 (46).

The PTHR2 assay displayed a greater sensitivity to α→β replacements than did the PTHR1 assay. For several single-β substitutions, the decline in agonist activity was so profound that an EC_{50} value could not be determined. In contrast to the findings with PTHR1, there was no position among the first eight residues of PTH(1–34)-NH_2 at which all three isomeric α→β replacements were well tolerated. At position 1, the (*S**)-β^2 and β^3-replacements have little effect on agonist potency, but the (*R**)-β^2 replacement causes a modest decline. At position 2, the (*S**)-β^2 replacement causes a modest activity decline, while the (*R**)-β^2 and β^3-replacements are well tolerated. At the remaining sites, the (*R**)-β^2 replacements are uniformly unfavorable in terms of agonist potency, while the impact of (*S**)-β^2 replacements is quite variable, ranging from very disruptive (position 4) to well tolerated (positions 6 and 7). β^3-Replacements at positions 3–8 of PTH(1–34)-NH_2 exert variable effects on

	Tolerance Pattern of β Amino Acid		
Position	(R*)-β²AA	(S*)-β²AA	(S*)-β³AA
1			
2			
3			
4			
5			
6			
7			
8			

Fig. 2. Tolerance pattern of β^2- and β^3-amino acids for PTHR1 and PTHR2 activation: green denotes potent PTHR1-selective agonism; yellow denotes potent PTHR2-selective agonism; purple denotes potent agonism of both PTHR1 and PTHR2; blank denotes poor agonism of both PTHR1 and PTHR2.

PTHR2 agonist activity as well, but the pattern differs from that manifested among the (S*)-β^2 replacements.

Enhanced Selectivity via Double α→β Replacement. Based on the β-scan results (*SI Appendix*, Fig. S1*B*), we hypothesized that it would be possible to design PTH(1–34)-NH_2 homologs containing α→β replacements at two sites in the N-terminal region that would display high selectivity for either PTHR1 activation or PTHR2 activation, in contrast to the potent activation of both receptors displayed by PTH(1–34)-NH_2 itself. We examined α/β-peptide **1**, which contains α→(R*)-β^2 replacements at positions 1 and 7, as a PTHR1-selective candidate. α/β-Peptide **2**, containing α→(R*)-β^2 replacement at position 2 and α→β^3 replacement at position 6, was evaluated as a PTHR2-selective candidate. The basis for these replacement choices is illustrated in Fig. 2.

For both **1** and **2**, the two α→β replacements function synergistically (Fig. 3). Neither replacement in **1**, on its own, causes a diminution of PTHR1 agonist activity (*SI Appendix*, Fig. S1*B*), and implementing the two replacements simultaneously also does not cause an activity decline. On the other hand, each of the replacements in **1** leads to significant decline in PTHR2 agonist activity (~sixfold and ~40-fold), and the pairing generates an α/β peptide that has almost no detectable activity for this receptor in this assay. For **2**, the α→β replacements individually cause slight increases in agonist activity at PTHR2, and the combination of these backbone modifications leads to further potency enhancement relative to PTH(1–34)-NH_2. On the other hand, each of the replacements in α/β-peptide **2** causes a significant decline in PTHR1 agonist potency (~10-fold and ~20-fold), and the combination leads to a more substantial potency decline (~100-fold). Moreover, **2** reaches a maximum PTHR1 activation level that is only ~80% of the maximum achieved by PTH(1–34)-NH_2.

Binding to PTHR1 and PTHR2. The selective agonism displayed by α/β-peptides **1** and **2** relative to PTH(1–34)-NH_2 could arise because of differences relative to PTH(1–34)-NH_2 in their affinities for PTHR1 and PTHR2, or because of differences in the abilities of **1** and **2** to activate each receptor upon binding. We conducted binding assays for PTHR1 and PTHR2 with these two PTH(1–34)-NH_2 analogs in an effort to distinguish these two possibilities (Fig. 3*A* and *SI Appendix*, Fig. S2). Two conformational states have been proposed for PTHR1 and for PTHR2, one that is G-protein dependent (RG) and another that is G-protein independent (R^0) (47). Distinct assays are available for binding to the R^0 and RG states for each receptor. For both PTHR1 and PTHR2, α/β-peptide **1** has higher RG affinity and lower R^0 affinity than does PTH(1–34)-NH_2. In contrast, α/β-peptide **2** has lower RG affinity than does PTH(1–34)-NH_2 for both receptors, but **2** and PTH(1–34)-NH_2 are comparable in terms of R^0 affinity for both receptors. The PTHR1/PTHR2 R^0 affinity ratios are very similar for **1**, **2**, and PTH(1–34)-NH_2, and the PTHR1/PTHR2 RG affinity ratios vary only by approximately sixfold among these three agonists.

Collectively, the results of the binding studies suggest that the selective agonism displayed by each of the α/β-peptides arises mainly from selective receptor activation rather than from selective binding to one receptor or the other. Further evidence that a backbone-modified PTH(1–34)-NH_2 homolog can maintain affinity for a receptor despite loss of agonist activity was obtained from the observation that α/β-peptide **1** functions as an antagonist of PTHR2 activation by PTH(1–34)-NH_2 (*SI Appendix*, Fig. S3). The conclusion that backbone-modified homologs of PTH(1–34)-NH_2 retain the ability to occupy the orthosteric site but are deficient in terms of inducing an active receptor conformation stands in contrast to previous analysis of naturally selective agonists PTHrP and TIP39, for which

A

	PTHR1				PTHR2			
Peptide	EC_{50} (nM)	E_{max}	R^0-IC_{50} (nM)	RG-IC_{50} (nM)	EC_{50} (nM)	E_{max}	R^0-IC_{50} (nM)	RG-IC_{50} (nM)
PTH(1-34)-NH_2	0.22 ± 0.04	100	6.95 ± 0.81	0.34 ± 0.06	1.07 ± 0.14	100	0.96 ± 0.48	0.63 ± 0.23
α/β-peptide 1	0.23 ± 0.04	100 ± 1	27.18 ± 7.22	0.13 ± 0.02	ND	ND	4.94 ± 0.81	0.04 ± 0.01
α/β-peptide 2	22.09 ± 1.50	79 ± 6	5.25 ± 1.64	0.80 ± 0.08	0.13 ± 0.01	104 ± 2	0.64 ± 0.03	0.84 ± 0.19

B

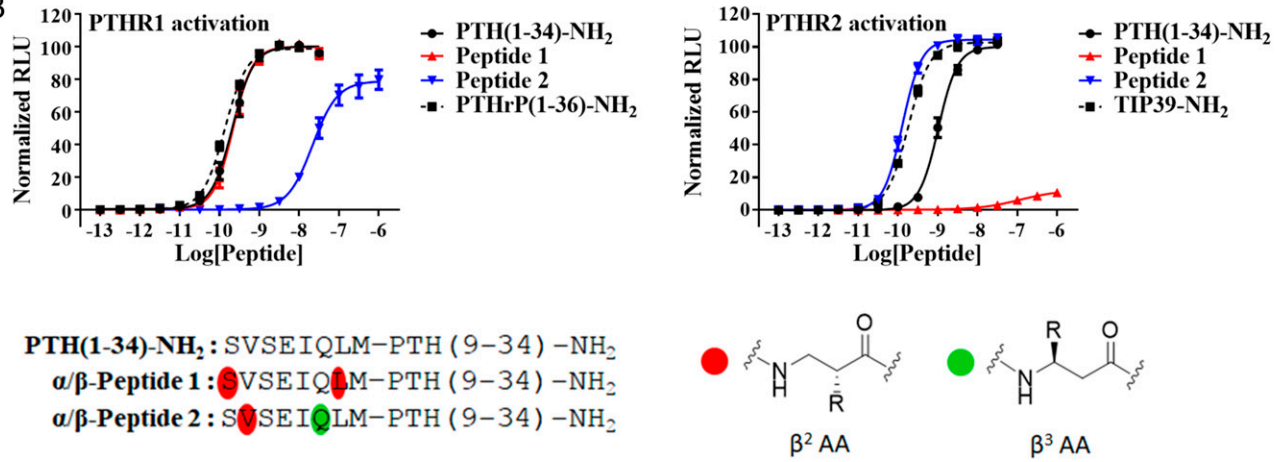
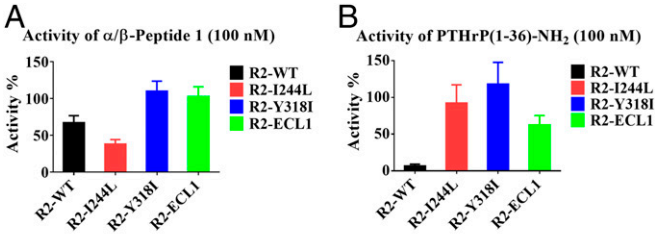


Fig. 3. Signaling and binding properties of PTHR1- and PTHR2-selective α/β-peptide analogs of PTH(1–34)-NH_2. (*A*) EC_{50}, E_{max}, and R^0 and RG IC_{50} values for PTH(1–34)-NH_2, α/β-peptide **1**, and α/β-peptide **2**. (*B*) Dose–response curves of PTHR1 and PTHR2 activation in HEK293 cells stably expressing PTHR1 or PTHR2. For PTHR1 activation and all of the binding data, data represent mean ± SEM from three independent measurements; for PTHR2 activation, data represent mean ± SEM from four independent measurements. Curves were fit to the data using a four-parameter sigmoidal dose–response equation.

selectivity in receptor binding plays a major role in determining agonist selectivity (8, 48).

The mechanistic origin of the selective activation displayed by α/β-peptides **1** and **2** is unclear at present, due to the lack of structural characterization of PTHR1 or PTHR2. Nevertheless, structural data for other class B GPCRs allow us to formulate a mechanistic hypothesis (49–51). The transmembrane (TM) domains of class B GPCRs adopt a unique "V-shaped" structure and present a ligand-binding pocket that is more open toward the extracellular side relative to the pockets of class A GPCRs (52). Cryo-EM structures of the GLP-1 receptor bound to GLP-1 (50) and the calcitonin receptor bound to calcitonin (51) show that the N-terminal residues of peptide agonists are inserted into the TM domain binding pocket. The agonist peptides appear to displace the extracellular ends of several TM helices and induce an outward movement of the intracellular part of TM helix 6, which creates a cavity on the cytoplasmic surface of the receptor for G-protein binding. We extrapolate from this structural model to speculate that backbone modification in the N-terminal region of PTH(1–34)-NH_2 alters the mode of agonist interaction with the TM domain of PTHR1, PTHR2, or both, relative to PTH(1–34)-NH_2 itself. This altered interaction influences the position of TM helix 6 in the activated complex and thereby affects G-protein coupling to the receptor. A given N-terminal backbone modification might exert differential effects on the G-protein coupling of PTHR1 vs. PTHR2, which could explain the observed receptor activation selectivity for α/β-peptides **1** and **2**.

Molecular Basis of PTHR1 vs. PTHR2 Selectivity. Previous receptor-mutation studies have identified sites within PTHR1 and PTHR2 that play critical roles in determining peptide agonist selectivity (53). For example, PTHrP(1–36)-NH_2 is a potent agonist of PTHR1 and a very weak agonist of PTHR2; however, modifications at three sites in PTHR2, based on residues found at analogous sites in PTHR1, rescue the agonist activity of PTHrP(1–36)-NH_2. Specifically, any of three modifications to the PTHR2 sequence, (*i*) replacement of residues 199–208 at the N-terminal side of extracellular loop 1 (ECL1) of PTHR2 with the corresponding segment of PTHR1, (*ii*) mutation of I244 to L, or (*iii*) mutation of Y318 to I, generates a PTHR2 variant that is much more susceptible to activation by PTHrP(1–36)-NH_2 relative to wild-type PTHR2 (Fig. 4*B* and *SI Appendix*, Fig. S4*B*). Evaluation of α/β-peptide **1** with this panel of three PTHR2 variants yields an activity profile (Fig. 4*A* and *SI Appendix*, Fig. S4*A*) that is distinct from the activity profile observed with PTHrP(1–36)-NH_2. At the level of receptor expression required to detect significant activity rescue for 100 nM PTHrP(1–36)-NH_2 at the three mutant receptors, substantial activity at wild-type PTHR2 is observed for 100 nM α/β-peptide **1**. Significant increases in agonist activity are observed for two of the PTHR2 variants relative to wild-type receptor (ECL1 chimera and Y318I), but a decrease is evident relative to wild-type receptor for the third PTHR2 variant, I244L. Detailed interpretation of these differences is not possible in the absence of atomic-resolution structural data for PTHR1 or PTHR2, but the distinct response profiles of PTHrP(1–36)-NH_2 and α/β-peptide **1** to this set of receptor variants, particularly I244L, suggest that the molecular determinants underlying the PTHR1 vs. PTHR2 selectivity are at least partially different between PTHrP(1–36)-NH_2 and α/β-peptide **1**.



	R2-WT	R2-ECL1	R2-I244L	R2-Y318I
α/β-Peptide 1	67 ± 10	103 ± 13	38* ± 6	110§,* ± 14
PTHrP(1-36)	7 ± 2	62* ± 13	92§,* ± 25	118§,* ± 30

Fig. 4. Effect of PTHR2 mutations on agonist activity. (*A*) Data for α/β-peptide **1** represent mean ± SEM from five independent experiments. (*B*) Data for PTHrP(1–36)-NH_2 represent mean ± SEM from three independent experiments. The ligand activity (100 nM) is measured in HEK293 cells transiently transfected with wild-type PTHR2 or mutants. The activity of α/β-peptide **1** or PTHrP on each receptor is normalized to that of TIP39 (10 nM). §, Statistically significant difference from WT-PTHR2 using one-way analysis of variance followed by Dunnett's test ($P < 0.05$). *, Statistically significant difference from WT-PTHR2 using two-tailed Student's *t* test ($P < 0.05$).

We asked whether reciprocal point mutations of PTHR1 could enhance the signaling activity of PTHR2-selective agonists. TIP39-NH_2 does not activate wild-type PTHR1 and appears to be a very weak agonist of mutants PTHR1-L289I and PTHR1-I363Y (*SI Appendix*, Fig. S5*B*). α/β-Peptide **2** deviates partially from this pattern (*SI Appendix*, Fig. S5*A*) in that PTHR1-I363Y is even less susceptible to activation by **2** than is wild-type PTHR1. Overall, our observations with receptor variants suggest that the response of PTHR1 and PTHR2 to agonists that are selective by virtue of side-chain identity [such as PTHrP(1–36)-NH_2 or TIP39-NH_2] involves a set of contact residues on the receptor that is partially distinct from those that mediate the response to agonists that are selective by virtue of backbone modification (such as α/β-peptides **1** and **2**). Thus, these data suggest that side-chain modification and backbone modification represent complementary approaches for altering the agonist properties of a given starting peptide.

Duration of Activation for PTHR1 and PTHR2. To assess the duration of PTHR1 and PTHR2 activation induced by α/β-peptides **1** and **2**, we examined the time course of cAMP production by each receptor after stimulation with an agonist and subsequent washing of the cells to remove unbound peptide (washout assay). PTH(1–34)-NH_2 and the naturally selective agonists PTHrP(1–36)-NH_2 and TIP39-NH_2 were used as controls (54). During the initial "ligand-on" phase, cells stably expressing PTHR1 or PTHR2 were stimulated with an agonist concentration corresponding to ~EC_{80}; the medium contained D-luciferin. The luminescence emission caused by cAMP production was monitored until each agonist reached maximum response (E_{max}; ~14 min in each case), at which point unbound peptide was washed away. After the addition of fresh medium containing D-luciferin, we monitored the luminescence decay. The area under the "ligand-off" curve (AUC) reflects the duration of signaling, which may be related to residence time of the agonist on the receptor but could have other origins at the molecular level. At PTHR1, the selective agonists α/β-peptide **1** and PTHrP(1–36)-NH_2 lead to more transient signaling (smaller AUC) than does PTH(1–34)-NH_2 (Fig. 5*A* and *SI Appendix*, Table S1A). At PTHR2, the selective agonists α/β-peptide **2** and TIP39-NH_2 induce more prolonged signaling than does PTH(1–34)-NH_2 (Fig. 5*B* and *SI Appendix*, Table S1B).

Vilardaga and coworkers (55, 56) discovered that PTH(1–34) induces sustained PTHR1 signaling from internalized receptors; endosomal acidification ultimately terminates signaling by destabilizing the ligand/receptor interaction. The short signal duration observed for α/β-peptide **1** led us to speculate that the interaction between **1** and PTHR1 might be highly sensitive toward endosomal acidification. To test this hypothesis, we studied the effect of bafilomycin A1 (BA1) (57), an inhibitor of endosomal acidification, on the signal duration of **1** (*SI Appendix*, Figs. S7*A* and S9*A*). BA1 enhances the signal duration of PTH(1–34)-NH_2, as previously reported (56), and we found that the signal durations of **1** and PTHrP(1–36)-NH_2 were enhanced even

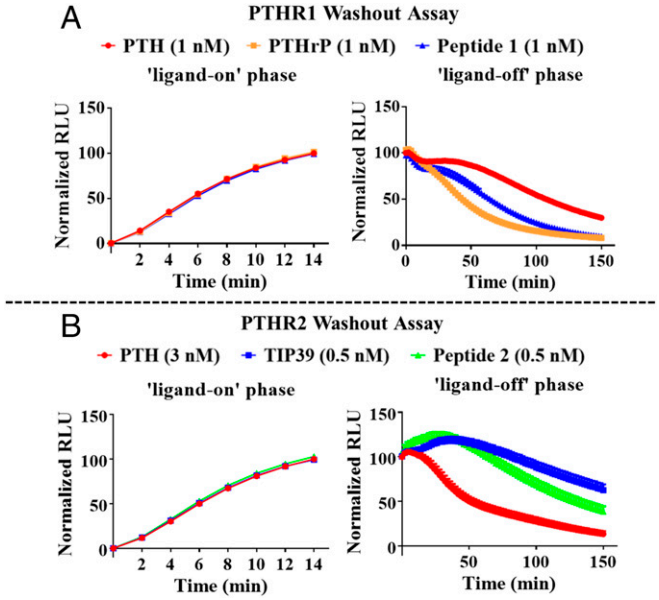


Fig. 5. PTHR1 and PTHR2 washout assays. (*A*) PTHR1 signaling durations of PTH(1–34)-NH_2, PTHrP(1–36)-NH_2, and α/β-peptide **1**. Data points represent mean ± SEM from 10 independent experiments. (*B*) PTHR2 signaling durations of PTH(1–34)-NH_2, TIP39-NH_2, and α/β-peptide **2**. Data points represent mean ± SEM from 11 independent experiments.

more significantly in the presence of BA1. Thus, despite sharing exactly the same sequence of side chains, **1** and PTH(1–34)-NH_2 appear to have differential sensitivity to endosomal acidification in terms of receptor engagement.

For PTHR2, BA1 appears to have very limited effect on the signal durations of PTH(1–34)-NH_2, TIP39, or α/β-peptide **2** (*SI Appendix*, Figs. S7*B* and S9*B*). We wondered whether PTHR2 activation induced by these agonists occurs primarily on the cell surface rather than after internalization. For GPCR agonists that induce endosomal signaling, receptor binding is considered to be pseudoirreversible because the agonist is physically prevented from moving far from the receptor (58). In this scenario, a bound ligand should not be very susceptible to a competitive antagonist that cannot cross cell membranes (59). Introduction of HYWH-TIP39 (60), a peptide that functions as a competitive PTHR2 antagonist, to the ligand-off phase of the PTHR2 washout assay significantly reduced (>60%) the signal durations of **2**, PTH(1–34)-NH_2, and TIP39-NH_2, which suggests that none of these agonists causes endosomal signaling (*SI Appendix*, Figs. S8*B* and S9*D*). The effect of competitive antagonism in the PTHR1 washout assay was examined using the known antagonist (D)Trp^{12},Tyr^{34}-bPTH(7–34) (61). In the presence of this antagonist, the signal duration of **1** was diminished by ∼60%, whereas that of PTH(1–34)-NH_2 was diminished by only ∼30% (*SI Appendix*, Figs. S8*A* and S9*C*). This difference in antagonist effect is consistent with the hypothesis that PTH(1–34)-NH_2 is more effective than α/β-peptide **1** at inducing endosomal signaling via the PTHR1.

Conclusions

All of the β-containing PTH(1–34)-NH_2 homologs described here contain exactly the same complement and sequence of side chains as is found in PTH(1–34)-NH_2 itself, with the exception of two cases in which the sulfur atom in the Met-8 side chain has been conservatively replaced by a methylene unit. Most of these homologs differ from PTH(1–34)-NH_2 by the presence of just one additional methylene unit in the backbone; homologs **1** and **2** each contain two additional methylene units relative to PTH(1–34)-NH_2. The data show that these limited backbone modifications can exert profound effects on agonist potency toward PTHR1, PTHR2, or both. The impact of these limited backbone modifications rivals that achieved by natural selection (as manifested in the activities of PTHrP and TIP39), which is constrained to exploration of side-chain variations. Our results further demonstrate that within a peptidic backbone, shifting a side-chain position by the length of a single carbon–carbon bond ($\beta^2 \rightarrow \beta^3$ or vice versa) can result in substantial changes in activity and/or selectivity. This discovery could not have been predicted and highlights the importance of considering β^2-homoamino-acid residues, despite the synthetic effort required to generate the necessary precursors.

Previous research has demonstrated that extensive backbone modification can substantially enhance the resistance of bioactive peptides to proteolysis (33, 34, 36, 38, 46). Only one or two β-amino acids are incorporated into the 34-residue peptides described here, and precedent suggests that this low level of modification will not have much impact on susceptibility to proteases. Since β-amino-acid residues can be used in polypeptide hormone analogs to adjust both proteolytic stability, as shown previously (46), and agonist selectivity, as shown here, it seems likely that backbone-modified analogs featuring both of these desirable attributes will ultimately be accessible.

Methods

Full details are provided in *SI Appendix*.

Peptide Synthesis. Peptides were synthesized by microwave-assisted solid-phase methods on NovaPEG Rink Amide resin. Full details on peptide synthesis, purification, and quantification can be found in *SI Appendix*.

Cell Culture and Glosensor cAMP Assay. GP2.3 cells are HEK293 cells stably expressing glosensor cAMP reporter and human PTHR1, and G2R cells are HEK293 cells stably expressing glosensor cAMP reporter and human PTHR2 (45). Both cell lines were cultured in DMEM supplemented with 10% FBS. Cells were seeded into 96-well Corning plate and used for cAMP assays after the cells had formed a confluent monolayer. Upon the removal of culture medium, the intact cells in 96-well plates were incubated in CO_2-independent medium containing D-luciferin (0.5 mM) for 20 min at room temperature. After this period, cells in each well were treated with peptides at various concentrations, and luminescence resulting from cAMP activation was measured for 30 min on a BioTek Synergy 2 plate reader. The peak luminescence signals (which usually appeared 14–20 min after peptide addition) were used to generate dose–response curves.

The dose–response curves were fit to the data by using the four-parameter sigmoidal dose–response equation in Prism 6.0. Reported EC_{50} and E_{max} values represent mean ± SEM of ≥3 independent measurements. All of the α/β-peptide analogs of PTH(1–34)-NH_2 with specific EC_{50} values (*SI Appendix*, Fig. S1*A*) reach a maximum response that is ≥80% of the maximum response observed for PTH(1–34)-NH_2 (*SI Appendix*, Fig. S1*C*). For PTHR2 activation, some α/β-peptide analogs of PTH(1–34)-NH_2 did not reach a maximum response at 1 μM. In these cases, the lower limits of the EC_{50} values are provided.

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