Autoactivation of Type-1 Parathyroid Hormone Receptors Containing a Tethered Ligand

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Interactions between the N-terminal residues of parathyroid hormone (PTH) and the region of the PTH receptor containing the extracellular loops and transmembrane domains are thought to be critical for receptor activation. We evaluated this hypothesis by replacing the large N-terminal extracellular domain of the human type 1 PTH receptor (hP1Re-WT) with residues 1–9 of PTH (AVSEIQLMH) using a tetraglycine linker between His-9 of the ligand and Glu-182 of the receptor near the extracellular terminus of transmembrane domain-1. Expression of this construct, hP1Re-Tether(1–9), in COS-7 cells resulted in basal cAMP levels that were 10-fold higher than those seen in control cells transfected with hP1Re-WT. Extending the ligand sequence to include Asn-10 and the activity-enhancing substitution of Leu-11 → Arg yielded hP1Re-[Arg11]Tether(1–11), for which we observed basal cAMP levels that were 50-fold higher than those seen with P1Re-WT. An alanine-scan analysis of hP1Re-[Arg11]Tether(1–11) revealed that Gln-6 and His-9 were not critical for autoactivation, whereas Val-2, Ile-5, and Met-8 were. The data show that tethered PTH/PTH can autoactivate. Analysis of the structure-activity relationships in these tethered receptor constructs can provide new information concerning how the N-terminal residues of PTH interact with the extracellular loops and transmembrane regions of the PTH-1 receptor, particularly in regard to receptor activation.

Human parathyroid hormone (hPTH) is an 84-amino acid polypeptide that binds to a class B G protein-coupled receptor, the PTH-1 receptor, and thereby plays a vital role in regulating the extracellular concentrations of ionized calcium (1). The PTH-1 receptor also mediates the actions of PTH-related peptide, a large (~140 amino acids) polypeptide ligand that plays a critical role in the developmental of several organs, particularly the skeleton (1). PTH has potent anabolic effects on bone in humans (2) and, thus, is a potential therapy for metabolic bone diseases, such as osteoporosis (3). Structure-activity analyses of PTH have shown that the 1–34 fragment is sufficient for full biological activity (4). Within the PTH(1–34) peptide, the N-terminal residues are the most critical for receptor activation, whereas the C-terminal residues are more important for determining receptor binding affinity (4, 5).

Recent receptor mutagenesis and photochemical cross-linking approaches suggest that residues within the C-terminal 15–34 region of PTH(1–34) interact with the relatively large (~170 amino acid) N-terminal extracellular domain of the PTH-1 receptor and that the N-terminal residues of PTH interact with the 7 transmembrane domains and extracellular loops of the receptor (6–10). In further support of the latter component of this hypothesis, we recently showed that a peptide as small as PTH(1–14) could stimulate cAMP formation, albeit weakly, with both the wild type PTH receptor and a truncated PTH receptor (P1Re-delNt) that lacked most of the N-terminal domain (11). The potency of PTH(1–34) was severely diminished with P1Re-delNt, as compared with its potency with the wild type receptor (EC_{50} values = ~500 nM and ~2 nM, respectively). In contrast, the potency of PTH(1–14) was equivalent with both the intact receptor and with P1Re-delNt (EC_{50} values = ~200 μM). An alanine-scanning analysis performed on PTH(1–14) revealed that residues (1–9) were critical for interacting with the heptahelical and extracellular loop region of the receptor (11).

Based on the above results, we considered the possibility that the first nine residues of PTH could be sufficient for receptor activation if they were constrained to within the region of the receptor containing the seven transmembrane domains and extracellular loops. As described herein, we show that this can be accomplished by tethering the N-terminal residues of PTH directly to a truncated receptor lacking the N-terminal extracellular domain. The resulting tethered ligand/receptor constructs are active and exhibit a similar, yet not identical, mutational profile to that seen previously with exogenous PTH(1–14) and PTH(1–34) peptide ligands. This system provides a new approach for analyzing how the N-terminal residues of PTH contribute to interactions with the PTH-1 receptor.

MATERIALS AND METHODS

Peptides—The peptides PTH(1–14) (rPTH(1–14)NH₂), rPTH(1–34) ([Nle³,10,Tyr²³]rPTH(1–34)NH₂), and Q-PTH(1–34) (Q-Ala₁,3,10,12,Arg⁻¹¹,Tyr²³)hPTH(1–34)NH₂ were prepared by the M. G. H. Biopolymer Synthesis Facility, Boston, MA, as described previously. The Q-PTH(1–34) analog contains a quartet (Q) of substitutions (Ser³,10 → Ala, Asn¹⁰ → Ala, Leu¹¹ → Arg, and Glb₁² → Ala) that strongly enhances potency on PTH-1 receptors lacking the N-terminal extracellular domain. This peptide is, thus, well suited for assessing the agonist responsiveness of the truncated receptors used in this study.

PTH Receptor Mutagenesis and Expression in COS-7 Cells—The pCDNA-1-based plasmid encoding the intact hPTH-1 receptor (13) (hP1Re-WT) was used as a starting plasmid for mutagenesis. The truncated human PTH-1 receptor (hP1Re-delNt) is deleted for residues 24 to 181 and was constructed from hP1Re-WT by oligonucleotide-directed mutagenesis (14). Signal peptide cleavage of this receptor is predicted to occur between Ala-22 and Tyr-23 (15) and, thus, leave
Tyr-23 as the N-terminal residue followed directly in sequence by Glu-182 located near the extracellular boundary of the first transmembrane helix. A similarly truncated rat PTH receptor containing a nine-amino acid N-terminal extracellular epitope tag described by us previously was expressed on the surface of COS-7 cells at 60% of the level of the intact wild-type receptor (11, 16). The tethered human PTH-1 receptor, hP1Rc-Tether(1–9), was made from hP1R-delNt by a single oligonucleotide-directed mutagenesis step that inserted a 13-amino acid sequence (AVSEIQLMHGGGGG) corresponding to PTH(1–9) joined C-terminally to a tetraglycine spacer between the Tyr-23/Glu-182 peptide bond. The predicted signal peptidase cleavage of this receptor between Ala-22 and Tyr-23 (15) generates the N-terminal tyrosine followed directly by Ala-1 of the ligand sequence (see Fig. 1). The receptors hP1Rc-Tether(1–10), hP1Rc-Tether(1–11), and hP1Rc-[Arg11]Tether(1–11) were made from hP1R-Tether(1–9) by oligonucleotide-directed mutagenesis. Transient transfections of COS-7 cells were performed using DEAE-dextran and cesium chloride-purified plasmid DNA, as described previously (16). All transfections utilized 200 ng of DNA/well of a 24-well plate, except for the experiments shown in Fig. 5, which utilized the amounts of DNA indicated in the graph.

CAMP Stimulation—Measurements of cAMP formation in COS-7 cells were performed in 24-well plates 4 days after transfection (11). The cells were rinsed with 0.5 ml of binding buffer (Dulbecco’s modified Eagle’s medium containing 2 mM NaCl, 5 mM KCl, 2 mM CaCl2, 5% heat-inactivated horse serum, 0.5% fetal bovine serum, adjusted to pH 7.7 with HCl) and treated with 200 μl of IBMX buffer (Dulbecco’s modified Eagle’s medium containing 2 mM 3-isobutyl-1-methylxanthine, 1 mg/ml bovine serum albumin, 35 mM Hepes-NaOH, pH 7.4) and 100 μl of binding buffer (basal) or binding buffer containing varying amounts of a PTH peptide analog (total volume = 300 μl). The cells were then incubated at room temperature for 1 h (or less for the time course experiment of Fig. 4), and the IBMX-containing buffer was removed. The cells were immediately frozen on crushed dry ice, and then 0.5 ml of 50 mM HCl was added, and the cells were refrozen on dry ice. The cAMP content of the thawed and diluted lysate was determined by radioimmunoassay.

Data Calculation—Calculations were performed using Microsoft Excel. For some experiments, a four-parameter nonlinear regression equation was used to fit curves to the cAMP dose-response data and to obtain the corresponding EC50 values (16). 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
To construct PTH receptors having the PTH ligand sequence covalently “tethered” to the body of the receptor, we utilized as a starting scaffold a human PTH-1 receptor analog that is deleted for most of the extracellular N-terminal domain, hP1Rc-delNt (11). This deletion mutant receptor retains the native signal sequence, as do each of the subsequent tethered receptor constructs, such that signal peptidase cleavage between the Ala-22 and Tyr-23 peptide bond is predicted to generate the mature receptor mutant with an N-terminal tyrosine residue (15). In hP1Rc-delNt, this tyrosine is joined directly to glutamate 182; in the tethered receptors, the PTH segment, linked C-terminally to a tetraglycine linker, is inserted between the N-terminal tyrosine and glutamate 182 (Fig. 1). The N-terminal tyrosine was not expected to be a major detriment to the potential signaling activity induced by the tethered PTH segment because [Tyr1]rPTH(1–14)NH2 was 50% as active as native PTH(1–14) in stimulating a cAMP response in cells expressing the P1Rc.3

The basal and ligand-stimulated signaling properties of the tethered PTH-1 receptors in transiently transfected COS-7 cells are shown in Fig. 2. In the absence of added agonist, cells expressing hP1Rc-Tether(1–9) exhibited basal cAMP levels that were 10-fold higher than those observed with hP1Rc-WT or hP1Rc-delNt (Fig. 2A). Extension of the ligand chain by one or two residues yielded hP1Rc-Tether(1–10) and hP1Rc-Tether(1–11), which produced moderate, but statistically significant (p < 0.005), improvements in the levels of basal cAMP signaling relative to hP1Rc-Tether(1–9). Basal cAMP signaling was increased substantially by the replacement of the native leucine at position 11 of hP1Rc-Tether(1–11) with arginine; we recently found that this same substitution enhances cAMP potency in short N-terminal PTH(1–14) analog peptides.4 Each of the tethered hP1Rc constructs responded to a 1 μM dose of exogenous PTH(1–34) analog to a similar extent (Fig. 2B).

To further evaluate the ability of these tethered receptors to respond to exogenous ligands, we performed dose-response analyses using rPTH(1–34), the Q-PTH(1–34) analog,2 and the N-terminal PTH(1–14) fragment (11). The receptor hP1Rc-Tether(1–9) exhibited agonist responses to these ligands that were much the same as those seen with hP1Rc-delNt (Fig. 3, B and C). With hP1Rc-[Arg11]Tether(1–11), only a weak stimulation of cAMP accumulation could be discerned with the full-length peptides, and no increase in cAMP accumulation above the already high basal level was detected for the shorter PTH(1–14) peptide (Fig. 3D).

The rate of cAMP accumulation induced by hP1Rc-[Arg11]Tether(1–11) was examined in a time-course experiment conducted in transfected COS-7 cells either in the absence or presence of a 1 μM dose of Q-PTH(1–34). In the absence of agonist ligand, cAMP accumulation increased rapidly following the addition of IBMX (t = 0) in cells expressing hP1Rc-[Arg11]Tether(1–11), such that within 5 min the cAMP level (78 ± 6 pmol/well) was nearly half the maximum level attained by the 40-min time point (156 ± 5 pmol/well, Fig. 4). This basal rate of cAMP accumulation observed with hP1Rc-[Arg11]Tether(1–11) was comparable with that observed with PTH(1–34)-treated cells expressing hP1Rc-WT and contrasted strongly with the untreated hP1Rc-WT-expressing cells, for which little or no cAMP accumulation was observed.

The basal and agonist-induced cAMP signaling activity of hP1Rc-[Arg11]Tether(1–11) was dependent on the amount of plasmid DNA used in the transient transfections of COS-7 cells

3 T. Gardella and P. H. Carter, unpublished observations.
The DNA-dependence of these responses were parallel to those seen with a constitutively active PTH-1 receptor that contained the His-223Arg point mutation at the cytoplasmic end of transmembrane helix 2 (17). At each equivalent DNA dose, the basal cAMP response observed with hP1Rc[Arg11]Tether(1–11) was approximately twice that observed for hP1Rc-H223R (range 1.6–2.3-fold).

The finding that the Leu-11Arg substitution substantially improved the signaling activity of both the tethered receptor construct and a synthetic PTH(1–14) peptide2 prompted us to investigate whether other similarities existed between the structure-activity profiles of free PTH peptides and the PTH portion of the tethered receptor. Thus, we performed an alanine scan analysis of the PTH residues 2–9 of hP1Rc[Arg11]Tether(1–11), because these amino acid positions correspond to the most critical functional sites in PTH(1–14) (11). As illustrated in Fig. 6A, position-specific effects of these alanine substitutions on the basal cAMP-signaling activity of hP1Rc-[Arg11]Tether(1–11) were observed. The alanine substitutions of glutamine 6 and histidine 9 had relatively mild effects on basal signaling. These results stand in contrast to the severe effects that alanine substitutions at positions 6 and 9 had on PTH(1–14) peptide activity (11). Alanine substitutions of glutamate 4 and leucine 7 had intermediate effects on the basal signaling of hP1Rc-[Arg11]Tether(1–11). The most severe reductions in basal activity occurred with the alanine substitutions of valine 2, isoleucine 5, and methionine 8. These strong reductions in activity paralleled the effects that substitutions at the corresponding positions in PTH(1–14) had on cAMP-signaling activity (11). Each of the alanine-substituted tethered receptors mediated a response to exogenous PTH(1–34) analog (1 μM) that was comparable with that observed with the unmodified hP1Rc-[Arg11]Tether(1–11) control receptor (Fig. 6B).
containing buffer (A) or IBMX buffer containing a 1 M dose of plasmid DNA encoding either hP1Rc-WT ( ), hP1Rc-[Arg11]Tether(1–11) ( ), or hP1Rc-H223R ( ), a constitutively active PTH-1 receptor containing a point mutation in transmembrane domain 2. Cells were assayed for intracellular cAMP accumulation after treatment for 60 min at room temperature with IBMX buffer alone (A) or IBMX buffer containing Q-PTH(1–34) ([Ala1,3,10,12Arg11,Tyr34]hPTH(1–34)amide) at a concentration of 1 μM (B). The data shown (mean ± S.E.) were combined from four separate experiments, each performed in duplicate. Some error bars are smaller than the symbol.

This study describes a series of novel tethered PTH ligand/PTH receptor constructs in which the N-terminal residues of the PTH ligand are fused to the body of the heptahelical G protein-coupled human PTH-1 receptor. This approach was pursued as a means to potentially simplify the analysis of the interactions between PTH and its receptor. This interaction can be otherwise difficult to evaluate, given the high degrees of freedom inherent to a bimolecular system involving large diffusible proteins of uncertain three-dimensional structures. Each of the tethered receptors in our study exhibited elevated basal cAMP signaling levels, as compared with hP1Rc-WT, when transiently expressed in COS-7 cells. The highest constitutive activity occurred with hP1Rc-[Arg11]Tether(1–11), for which the basal cAMP levels were 75% of the maximum cAMP response attained by PTH(1–34)-treated hP1Rc-WT. The basal signaling activities of the tethered receptors were dependent on the amount of DNA used for the COS-7 cell transfection and followed a time course that closely resembled that observed for the agonist-stimulated hP1Rc-WT. The basal signaling of hP1Rc-[Arg11]Tether(1–11) resulted in approximately twice the level of intracellular cAMP that was obtained with a previously described constitutively active PTH-1 receptor, hP1Rc-H223R (17), when the two receptors were transfected using equal amounts of plasmid DNA. The ability of a small peptide derived from the activation domain of PTH to stimulate G protein coupling when tethered to the body of the PTH receptor bears direct similarity to the intramolecular mechanism of activation utilized by the protease-activated receptors, such as the well characterized thrombin receptor (18).

For the tethered PTH receptors of the present study, the native ligand sequences of PTH(1–9), PTH(1–10), and PTH(1–11) were weaker than the tethered PTH(1–11) sequence containing the Leu11 → Arg substitution, even though each of these tethered ligands was present at the same equimolar ratio, relative to the concentration of the membrane-embedded portion of the receptor. The level of expression of these receptors was likely to be comparable, given that each stimulated similar maximum levels of cAMP in response to high doses of an exogenous PTH(1–34) analog. There was not a simple correlation between PTH chain length and basal activity because hP1Rc-Tether(1–11) was statistically weaker than hP1Rc-Tether(1–10) (34 ± 1 and 52 ± 2 pmol/well, respectively, p < 0.001). The improved basal signaling of the Arg-11-containing tethered ligand is consistent with the favorable effect that this substitution had on the potency of PTH(1–11) and -(1–14) synthetic peptide analogs. This observation supports the hypothesis that the tethered PTH ligands utilize the same contact points for activating the receptor as do exogenous PTH peptide ligands.

We examined the above hypothesis further by performing an alanine-scan analysis of the PTH (2–9) segment of hP1Rc-[Arg11]Tether(1–11). The results revealed some differences from the alanine-scan data obtained from studies on the synthetic PTH (1–14) peptide (11), but there were important similarities to the prior study. In the case of PTH(1–14), the Ser-3 → Ala mutation produced a peptide that was 10% more active than native PTH(1–14), and Ala substitution at any other position in the (2–9) region reduced PTH(1–14) activity to approximately basal levels (position 1 is alanine in the native sequence). In the case of hP1Rc-[Arg11]Tether(1–11), alanine...

FIG. 5. DNA dependence of cAMP signaling by tethered and H223R constitutively active PTH-1 receptors. COS-7 cells were transiently transfected with the indicated dose of plasmid DNA encoding either hP1Rc-WT ( ), hP1Rc-[Arg11]Tether(1–11) ( ), or hP1Rc-H223R ( ), a constitutively active PTH-1 receptor containing a point mutation in transmembrane domain 2. Cells were assayed for intracellular cAMP accumulation after treatment for 60 min at room temperature with IBMX buffer alone (A) or IBMX buffer containing Q-PTH(1–34) ([Ala1,3,10,12Arg11,Tyr34]hPTH(1–34)amide) at a concentration of 1 μM (B). The data shown (mean ± S.E.) were combined from three separate experiments, each performed in duplicate. Some error bars are smaller than the symbol.

FIG. 6. Alanine-scan of the PTH(2–9) region of hP1Rc-[Arg11]Tether(1–11). Residues 2 through 9 of the PTH component of hP1Rc-[Arg11]Tether(1–11) were individually replaced by alanine, and the resulting mutant receptors were transiently transfected into COS-7 cells. The unsubstituted hP1Rc-[Arg11]Tether(1–11) receptor served as a control. After treatment for 60 min at room temperature with IBMX-containing buffer (A) or IBMX buffer containing a 1 μM dose of Q-PTH(1–34) (B), the intracellular cAMP levels were determined. The data shown (mean ± S.E.) were combined from three separate experiments, each performed in duplicate.

DISCUSSION

This study describes a series of novel tethered PTH ligand/PTH-1 receptor constructs in which the N-terminal residues of the PTH ligand are fused to the body of the heptahelical G protein-coupled human PTH-1 receptor. This approach was pursued as a means to potentially simplify the analysis of the interactions between PTH and its receptor. This interaction can be otherwise difficult to evaluate, given the high degrees of freedom inherent to a bimolecular system involving large diffusible proteins of uncertain three-dimensional structures. Each of the tethered receptors in our study exhibited elevated basal cAMP signaling levels, as compared with hP1Rc-WT, when transiently expressed in COS-7 cells. The highest constitutive activity occurred with hP1Rc-[Arg11]Tether(1–11), for which the basal cAMP levels were 75% of the maximum cAMP response attained by PTH(1–34)-treated hP1Rc-WT. The basal signaling activities of the tethered receptors were dependent on the amount of DNA used for the COS-7 cell transfection and followed a time course that closely resembled that observed for the agonist-stimulated hP1Rc-WT. The basal signaling of hP1Rc-[Arg11]Tether(1–11) resulted in approximately twice the level of intracellular cAMP that was obtained with a previously described constitutively active PTH-1 receptor, hP1Rc-H223R (17), when the two receptors were transfected using equal amounts of plasmid DNA. The ability of a small peptide derived from the activation domain of PTH to stimulate G protein coupling when tethered to the body of the PTH receptor bears direct similarity to the intramolecular mechanism of activation utilized by the protease-activated receptors, such as the well characterized thrombin receptor (18).

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substitution of Ser-3 yielded a mutant that resulted in 23% less cAMP accumulation than did the unsubstituted control tethered receptor. Substitutions of alanine at Gln-6 and His-9 yielded tethered receptor mutants that were nearly as active as hP1Rc-[Arg11]Tether(1–11) (basal cAMP levels were 83% and 67% of control, respectively, Fig. 6). Perhaps more importantly, alanine substitutions at Val-2, Ile-5, and Met-8 yielded receptors with markedly reduced basal cAMP signaling activity. These results on residues 2, 5, and 8 correlate closely with previous functional studies of PTH(1–14) (11) as well as PTH(1–34)- or PTHrP (1–36)-length analog peptides (19–21) that have demonstrated the importance of these three residues in mediating a productive interaction with the PTH-1 receptor. A recent computer modeling study has predicted that Ile-5 and Met-8 of PTH penetrate the heptahelical core of the receptor, whereas Val-2 interacts with extracellular loop 3 (22). The reasons for the greater mutational tolerance that we observed for certain PTH residues in the context of hP1Rc-[Arg11]Tether(1–11) (basal cAMP levels were 83% and 67% of control, respectively, Fig. 6). Perhaps more importantly, alanine substitutions at Val-2, Ile-5, and Met-8 yielded receptors with markedly reduced basal cAMP signaling activity. These results on residues 2, 5, and 8 correlate closely with previous functional studies of PTH(1–14) (11) as well as PTH(1–34)- or PTHrP (1–36)-length analog peptides (19–21) that have demonstrated the importance of these three residues in mediating a productive interaction with the PTH-1 receptor. A recent computer modeling study has predicted that Ile-5 and Met-8 of PTH penetrate the heptahelical core of the receptor, whereas Val-2 interacts with extracellular loop 3 (22). The results of several mutational analyses are in general support of these predictions, as residues within transmembrane domains 2, 5, 6, and 7 of the PTH-1 receptor have been shown to contribute importantly to PTH-induced cAMP-signaling responses (19, 23, 24), and residues in or near the third extracellular loop have been implicated as interaction sites for residues 1–2 of PTH (7, 19). Recent cross-linking data support such a receptor location for the N-terminal residues of PTH (8, 10). We are presently investigating the influence of mutations in these and other (25, 26) receptor domains on the constitutive signaling activity of the tethered receptor constructs.

The results presented in this report suggest that the tethered ligand system can offer new insights into the mechanism by which the N-terminal residues of PTH interact with the PTH-1 receptor and induce transmembrane signaling. The information from these and future studies on the tethered receptors could help to constrain the emerging three-dimensional models of the PTH/PTH-1 receptor complex. The dramatic minimization of the bioactive ligand sequence that is now possible due to the elimination of the need for high affinity binding should simplify such analyses and could lead to a better definition of the minimal pharmacophore required for PTH-1 receptor activation.

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