Evaluating the Signal Transduction Mechanism of the Parathyroid Hormone 1 Receptor

EFFECT OF RECEPTOR-G-PROTEIN INTERACTION ON THE LIGAND BINDING MECHANISM AND RECEPTOR CONFORMATION

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Ligand binding to the PTH1 receptor is described by a “two-site” model, in which the C-terminal portion of the ligand interacts with the N-terminal domain of the receptor (N interaction), and the N-terminal region of the ligand binds the juxtamembrane domain of the receptor (J interaction). Previous studies have not considered the dynamic nature of receptor conformation in ligand binding and receptor activation. In this study the ligand binding mechanism was compared for the G-protein-coupled (RG) and uncoupled (R) PTH1 receptor conformations. The two-site model was confirmed by demonstration of spatially distinct binding sites for PTH(3–34) and PTH(1–14); PTH(1–14), which binds predominately to the J domain, only partially inhibited binding of 125I-PTH(3–34); and PTH(3–34), shown to bind predominantly to the N domain, only partially inhibited PTH(1–14)-stimulated cAMP accumulation. To assess the effect of R-G coupling, ligand binding to R was measured by displacement of 125I-PTH(3–34) with 30 μM guanosine 5’-3-O-(thio)triphosphate (GTPγS) present, and binding to RG was measured by displacement of 125I-[MAP]PTHrP(1–36) (where MAP is model amphipathic peptide), a new radioligand that binds selectively to RG. Agonists bound with higher affinity to RG than R, whereas antagonists bound similarly to these states. The J interaction was responsible for enhanced agonist binding to RG: residues 1 and 2 were required for increased PTH(1–34) affinity for RG; residue 5 of MAP-PTHrP(1–36) was a determinant of R/G binding selectivity, and PTH(1–14) bound selectively to RG. The N interaction was insensitive to R-G coupling; PTH(3–34) binding was GTPγS-insensitive. Finally, several observations suggest the receptor conformation is more “closed” at RG than R. At the R state, an open conformation is suggested by the simultaneous binding of PTH(1–14) and PTH(3–34). At RG PTH(1–14) better occluded binding of 125I-PTH(3–34) and agonist ligands bound pseudo-irreversibly, suggesting a more closed conformation of this receptor state. The results extend the two-site model to take into account R and RG conformations and suggest a model for differences of receptor conformation between these states.

The parathyroid hormone 1 (PTH1)1 receptor is a cell-surface signal transducer for PTH and PTH-related protein (PTHrP). PTH plays a central role in calcium homeostasis; the hormone acts on target cells in bone (osteoblasts) and kidney (renal tubule cells) to increase blood calcium levels (1). PTHrP is an autocrine factor, believed to be involved in the maintenance of numerous tissues, and an important developmental regulator, controlling breast, pancreas, skin, and bone development (2, 3). PTH and PTHrP are involved in the etiology and treatment of disease. PTH, when administered intermittently, acts as a bone anabolic agent potentially useful for the treatment of osteoporosis (4). PTHrP is overproduced by certain tumors, leading to hypercalcemia through activation of the PTH1 receptor (5). The intracellular signaling pathways activated by PTH and PTHrP via the PTH1 receptor include stimulation of adenyl cyclase, increases of intracellular calcium, and activation of phospholipase C and phospholipase D (6–10).

Owing to its important physiological, pathophysiological, and therapeutic roles, the molecular mechanisms of PTH1 receptor function have been studied extensively (11). The receptor belongs to the type II family of G-protein-coupled receptors (GPCRs), which respond to peptide ligands of intermediate size such as secretin, glucagon, calcitonin, corticotropin-releasing hormone, and vasoactive intestinal polypeptide. The receptor can be divided into two functional domains; the large extracellular N-terminal domain (N domain) has been proposed to provide most of the binding energy for receptor-ligand interaction (12, 13), and the remaining juxtamembrane region of the receptor (J domain) is a determinant of receptor activation and second messenger generation (12–15). Likewise the ligand (PTH or PTHrP) can be divided into two binding regions; the 15–34 portion is a determinant of receptor binding affinity (12, 16, 17), and the 1–14 portion is a determinant of receptor activation for stimulation of cAMP production (12, 18–21). The cAMP-stimulating activity and high affinity binding of PTH and PTHrP are retained within an N-terminal fragment of 34 residues (22). These observations suggested a “two-site” mode of receptor-ligand interaction (Fig. 1), in which the C-terminal portion of the ligand interacts with the N domain of the receptor (N interaction), and the N-terminal ligand region binds to the J domain of the receptor (J interaction) (11–13, 19). This model has also been demonstrated for other type II GPCRs.

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1 The abbreviations used are: PTH, parathyroid hormone; PTHrP, PTH-related protein; b, bovine; h, human; r, rat; MAP, model amphipathic peptide; TIP(7–39), tuberoinfundibular peptide (7–39); G, G-protein; GPCR, G-protein-coupled receptor; R, G-protein-uncoupled receptor state; RG, G-protein-coupled receptor state; GDPγS, guanosine 5’-2-O-(thio)diphosphate; GTPγS, guanosine 5’-3-O-(thio)triphosphate.
(23–26). For the PTH1 receptor, this low resolution molecular model is supported by a large number of receptor manipulation and photochemical cross-linking studies, which have also suggested points of contact and/or proximity between specific amino acid side chains of the ligand and the receptor (14, 15, 27, 28, 30–32). These observations have been combined with ligand structure data (33, 34) and computer models of the receptor to provide atomic resolution structural models of certain regions of receptor-ligand interaction (28, 33, 35, 36).

Receptor-ligand interaction models for the PTH1 receptor have not taken into account the dynamic nature of receptor conformation. Conformational change is central to the ability of a GPCR to transduce the extracellular signal of ligand binding across the plasma membrane to the intracellular signal of G-protein activation (37, 38). As a result, evaluating the effect of this receptor conformational change is essential for understanding ligand binding and signal transduction mechanisms (37, 38). These mechanisms have been examined extensively for GPCRs of the type I family, such as the β2-adrenergic receptor, leading to the development of the ternary complex model and its extended variants (37–39). These models describe the reciprocal effects of G-protein (G) and agonist on their binding to the receptor (R). For type II GPCRs a great deal is known regarding the orientation of ligand binding, but very little is known regarding how the two-site binding mechanism is affected by the conformational changes in the receptor that result from R-G interaction. In this study we evaluated the effect of R-G interaction on the two-site binding mechanism for that result from R-G interaction. In this study we evaluated the mechanism is affected by the conformational changes in the receptor.

**EXPERIMENTAL PROCEDURES**

**Reagents and Peptides—**The following peptides were purchased from Bachem (Torrance, CA) or Peninsula Laboratories (Belmont, CA): [Nle<sup>8,18</sup>, Tyr<sup>36</sup>]bPTH(1–34)-amide, [Nle<sup>8,18</sup>, Tyr<sup>34</sup>]bPTH(3–34)-amide, [Nle<sup>8,18</sup>, Tyr<sup>34</sup>]hPTH(7–34)-amide, [Asp<sup>13</sup>, Tyr<sup>34</sup>]bPTH(7–34)-amide, [Nle<sup>8,18</sup>, Tyr<sup>34</sup>]hPTH(7–34)-amide, and [Tyr<sup>34</sup>]<sup>36</sup> bTPR(3–36)-amide. hPTH(1–34) and bTPR(3–34) were obtained from AnaSpec Inc. (San Jose, CA). bTIP(7–39) was obtained from Biomolecules Midwest (Waterloo, IL). [MAP<sup>22–31</sup>, Tyr<sup>36</sup>]PTHrP(1–36) and [MAP<sup>22–31</sup>, Tyr<sup>36</sup>]bPTHrP(1–36) were obtained from AnaSpec Inc. (San Jose, CA). [MAP<sup>22–31</sup>, Tyr<sup>36</sup>]bPTHrP(1–36) was obtained from ICN Biomedicals (Costa Mesa, CA). Cell culture supplies were obtained from Life Technologies, Inc., except for Dulbecco’s modified Eagle’s medium which was from Mediatech (Herndon, VA).

**Preparation of Radioligands—**The radioligands [Nle<sup>8,18</sup>, Tyr<sup>36</sup>]bPTH(1–34), [Nle<sup>8,18</sup>, Tyr<sup>34</sup>]bPTH(3–34) and [MAP<sup>22–31</sup>, Tyr<sup>36</sup>]bTPR(P1–36) were prepared by photochemical cross-linking studies, which have also suggested points of contact and/or proximity between specific amino acid side chains of the ligand and the receptor (14, 15, 27, 28, 30–32). The DNA was mixed before addition of DEAE-dextran. The G-proteins were wild-type Go<sub>a</sub> in pCDNA/Amp and a mutant G-protein, Go<sub>a(36β)</sub>, in which five residues in rat Go<sub>a</sub> were substituted for residues in the corresponding positions of rat Go<sub>a</sub> (N271K/R274D/R280K/T284D/I285T), also in pCDNA/Amp (43). The mutations decrease the ability of the G-protein to couple to the adenyl cyclase but increase the affinity of receptor for G-protein (43). For the experiment in Fig. 3B, COS-7 cells were transfected in 24-well plates with 200 μg of pCDNA.1 plasmid DNA encoding the human PTH1 receptor or a human PTH1 receptor from which residues 24–181 had been removed (PTHI<sub>1NT</sub> (21)). Previously described methods were used to isolate cell membranes from HEK293 cells and COS-7 cells (44, 45). Membrane protein was quantified using the copper bicinechonic acid method (Pierce) with bovine serum albumin as the standard.

**Measurement of cAMP Accumulation—**Previously described methods were used to measure cAMP accumulation in HEK293 cells expressing the PTH1 receptor (42) and in COS-7 cells expressing the PTH1 or PTHI<sub>1NT</sub> receptors (21). For HEK293 cell membranes the total amount of PTH1 receptor present in the assay varied from 20 to 66 μg, whereas for COS-7 membranes the level varied from 90 to 180 μg. Total binding was less than 15% of the number of counts added in all cases. Nonspecific binding was measured by inclusion of a large excess of the unlabeled analogue of the radioligand (300 nM for PTH(3–34) and 1.00 μM for [MAP<sup>22–31</sup>]bPTHrP(1–36)). The amount of radioligand added varied from 70 to 220 pM for [125I]<sup>2</sup>[Nle<sup>8,18</sup>, Tyr<sup>36</sup>]bPTH(3–34) and from 90 to 220 pM for [125I]<sup>2</sup>[MAP<sup>22–31</sup>, Tyr<sup>36</sup>]bPTHrP(1–36). When GTPγS was added to the assay, it was dispensed prior to the radioligands. In [125I]<sup>2</sup>[MAP<sup>22–31</sup>]bPTHrP(P1–36) dissociation experiments, radioligand and membranes were equilibrated for 3 h at 21 °C prior to the addition of a large excess of the unlabeled analogue of the radioligand (1.00 μM for [MAP<sup>22–31</sup>]bPTHrP(P1–36)) and 1.00 μM for [MAP<sup>22–31</sup>]bPTHrP(P1–36).

**Data Analysis—**Radioligand binding data were analyzed by nonlinear regression using Prism 2.01 (GraphPad Software Inc. San Diego, CA). Radioligand saturation of the PTH1 receptor was analyzed using a single affinity state saturation equation. Displacement of radioligand binding to the PTH1 receptor was analyzed using a single affinity state model (Equation 1) or a two affinity state model (Equation 2),

\[ Y = NSB + (SB + 1 + 10^{x - IC_{50}}) \]

where Y is the total counts/min bound in the presence of the competing ligand, NSB is nonspecific binding; SB is specific binding in the absence of the competing ligand, and X is the logarithm of the unlabeled ligand concentration.

\[ Y = NSB + (SB + 1)(1 + 10^{x - IC_{50}}) \]

(Eq. 2) where IC<sub>50</sub>, 1 and IC<sub>50</sub>, 2 are the IC<sub>50</sub> values for displacement of radioligand binding to affinity states 1 and 2, respectively, and F1 is the fraction of specific radioligand binding displaced from affinity state 1. The best fit was evaluated by comparing the goodness of fit for Equations 1 and 2 using a partial F test. The IC<sub>50</sub>, 1 values were corrected for the radioligand concentration (to obtain a measure of K<sub>I</sub>) using the method of Cheng and Prusoff (44). For the experiment in Fig. 3B, COS-7 cells were transfected in 24-well plates with 200 μg of pCDNA.1 plasmid DNA encoding the human PTH1 receptor or a human PTH1 receptor from which residues 24–181 had been removed (PTHI<sub>1NT</sub> (21)). Previously described methods were used to isolate cell membranes from HEK293 cells and COS-7 cells (44, 45). Membrane protein was quantified using the copper bicinechonic acid method (Pierce) with bovine serum albumin as the standard.
ligands can bind simultaneously to the receptor, according to Model 1 below:

\[
\begin{align*}
R + L(3-34) + L(1-14) & \xrightarrow{K_{1-14}} RL(3-34) + L(1-14) \\
R + L(3-34) & \xrightarrow{K_{3-34}} RL(3-34) + L(1-14)
\end{align*}
\]

where \( R \) is the receptor; \( L_{3-34} \) is the equilibrium association constant for \( ^{125}\text{I}-\text{PTH(3–34)} \); \( K_{3-34} \) is the concentration of \( ^{125}\text{I}-\text{PTH(3–34)} \); \( K_{1-14} \) is the equilibrium association constant for \( ^{125}\text{I}-\text{PTH(1–14)} \); \( \alpha \) is the cooperativity factor defining the effect of \( L_{1-14} \) occupancy on the receptor binding affinity of \( L_{3-34} \) and reciprocally the effect of \( L_{3-34} \) occupancy on the receptor binding affinity of \( L_{1-14} \). Binding of \( ^{125}\text{I}-\text{PTH(3–34)} \) in the presence of PTH(1–14) is described by Equation 3, as the fitted value of the lower plateau of the concentration dependence curve, Fig. 2A.

\[
Y = \text{NSB} + (1 + K_{3-34} L_{3-34} + L_{1-14} \alpha K_{1-14} X) / (1 + K_{1-14} X + K_{3-34} L_{3-34} + L_{1-14} \alpha K_{1-14} X) \tag{Eq. 3}
\]

where \( Y \) is the total \( ^{125}\text{I}-\text{PTH(3–34)} \) bound (cpm) in the presence of PTH(1–14); NSB is nonspecific binding; \( SB \) is specific \( ^{125}\text{I}-\text{PTH(3–34)} \) binding in the absence of PTH(1–14); \( L_{3-34} \) is the concentration of \( ^{125}\text{I}-\text{PTH(3–34)} \); and \( X \) is the concentration of PTH(1–14). A value of \( \alpha < 1 \) denotes negative cooperativity, in which binding of PTH(1–14) blocks binding of \( ^{125}\text{I}-\text{PTH(3–34)} \). If the value of \( \alpha \) is not greatly less than unity, it is possible that the level of inhibition of \( ^{125}\text{I}-\text{PTH(3–34)} \) binding produced by PTH(1–14) may not reach 100%. (Two other possibilities not observed in this study include "neutral" cooperativity, in which binding of the unlabeled ligand does not affect the level of binding of the radioligand, and positive cooperativity, in which the unlabeled ligand increases the level of radioligand binding.) This model is mathematically identical to that proposed for allosteric regulation of radioligand binding to muscarinic acetylcholine receptors by gallamine and other modulators and is described in detail in Ref. 52.

Statistical comparison of multiple means was performed by single factor analysis of variance. Statistical comparison of two means was performed using a two-tailed Student’s t test. Unless otherwise stated, data points in figures are presented as the mean ± S.E. of triplicate measurements, and in some cases the error bars are enclosed within the symbol.

RESULTS

Characterization of \( ^{125}\text{I}-(\text{MAP})\text{PTHrP(1–36)} \), a Novel Radioligand Selective for the RG State of the PTH1 Receptor—To evaluate the effects of receptor-G-protein coupling on the mechanism of ligand binding to the PTH1 receptor, assays were developed for measuring the equilibrium binding affinity of ligands for the uncoupled receptor (R) and the receptor-G-protein complex (RG). For the R state, the binding affinity of unlabeled ligands was measured by displacement of the agonist radioligand \( ^{125}\text{I}-(\text{Nle}^8,18,\text{Tyr}^{24}\text{Phe}^{34}\text{Phe})\text{PTH(3–34)} \) binding. Binding was measured in the presence of a high concentration (30 μM) of GTPγS, which binds to the G-protein and uncouples it from the receptor (47). (Binding of \( ^{125}\text{I}-\text{PTH(3–34)} \) is insensitive to GTPγS (48).) For the RG state a radioligand was required that bound selectively to the RG complex. We previously showed that the PTHrP analogue RS-66271 (40) displays a considerable selectivity for the RG state over the R state (44, 49) so we prepared a radiolabeled analogue of this peptide, \( ^{125}\text{I}-(\text{MAP})\text{PTH(1–36)} \). Membranes prepared from HEK293 cells stably expressing the PTH1 receptor (HEK293PTH1 membranes) exhibited specific high affinity \( ^{125}\text{I}-(\text{MAP})\text{PTH(1–36)} \) binding with a total binding:non-specific binding ratio of 5:1. No specific binding was detected in membranes prepared from nontransfected HEK293 cells or cells transfected with the human PTH2 receptor. Specific binding was also detected in membranes isolated from COS-7 cells expressing the human or rat PTH1 receptor and from ROS

![Fig. 1. Two-site model of receptor-ligand interaction for the PTH1 receptor.](image)
also evaluated by overexpression of G-protein. COS-7 cells were transfected with the PTH1 receptor alone or cotransfected with G-protein. Cotransfection with wild-type rat Goα resulted in a slight increase of 125I-[MAP]PTHrP(1–36) binding, but the total receptor level (labeled with 125I-PTH(3–34)) was considerably reduced (Fig. 2D). This reduction of receptor expression probably resulted from cAMP-dependent down-regulation of the receptor; overexpression of Goα resulted in a decrease of the basal cAMP level in COS-7 cells (from 0.84 ± 0.04 to 2.28 ± 0.01 pmol/mg). In an attempt to minimize this reduction of receptor expression, we used a mutant G-protein (Goα(α/β)) (43) that is impaired in adenylyl cyclase activation. This G-protein mutant also displays higher receptor binding affinity, which stabilizes high affinity binding of agonist ligands. Overexpression of Goα(α/β) did not increase the basal cAMP level in COS-7 cells (0.68 ± 0.04 pmol/mg). Coexpression with the PTH1 receptor resulted in a large increase of 125I-[MAP]PTHrP(1–36) binding with only a slight reduction of the level of receptor expression, as measured by the level of 125I-PTH(3–34) binding (Fig. 2D). This additional 125I-[MAP]PTHrP(1–36) binding represents the agonist high affinity RG state. Expression of Goα(α/β) increases the fraction of [MAP]PTHrP(1–36) binding in the high affinity state, and this component is sensitive to GTPγS (Fig. 8A).

Saturation binding experiments indicated that 125I-[MAP]PTHrP(1–36) bound with high affinity to the PTH1 receptor in HEK293PTH1 membranes (1.9 ± 0.8 nm, Fig. 2B). The sites recognized by this radioligand (Bmax = 180 ± 50 fmol/mg) represented only a fraction of the sites recognized by 125I-PTH(3–34) (820 ± 90 fmol/mg), indicating that only a fraction (22%) of the total receptor population is coupled to G-protein in HEK293PTH1 membranes (Fig. 2B). Radioligand association experiments demonstrated a slow rate of association of 125I-[MAP]PTHrP(1–36) (t1/2 of 1 h). Dissociation of the radioligand was complex (Fig. 2C). Three phases were detected in the absence of GTPγS as follows: a very rapid phase (t1/2 < 10 s, representing 18 ± 2% of specific binding), a more slowly dissociating phase (t1/2 = 47 ± 1 min, 50 ± 1% of binding), and a pseudo-irreversible phase (32 ± 2% of binding). 30 μM GTPγS removed most of the pseudo-irreversible component (now representing 4 ± 1% of specific binding), reduced the fraction of binding and t1/2 of the slower phase (30 ± 0%, 29 ± 1 min), and increased the amount of binding dissociating at the very rapid rate (to 66 ± 1%). These data suggest that GTPγS converts the
Comparison of Ligand Binding Affinity for R and RG States of the PTH1 Receptor—The effect of RG coupling on ligand binding to the PTH1 receptor was determined by measuring the affinity of unlabeled ligands for the R and RG states of the receptor in HEK293PTH1 membranes. Ligand binding affinity for the uncoupled receptor was measured by displacement of $^{125}$I-PTH(3–34) in the presence of 30 μM GTPγS. (This concentration of GTPγS was near-saturating for removal of the RG state. It reduced specific binding of $^{125}$I- [MAP]PTHrP(1–36) binding by 77%, close to the lower plateau value of the displacement curve in Fig. 2A of 78%). Ligand binding to the RG state was measured by displacement of $^{125}$I-[MAP]PTHrP(1–36) in the absence of exogenous guanine nucleotides. It is important to note that this assay provides only an approximate measurement of ligand affinity for RG, since a fraction of specific $^{125}$I-[MAP]PTHrP(1–36) binding is insensitive to guanine nucleotides (Fig. 2A), and a fraction of binding is pseudo-irreversible (Fig. 2C).

All agonist ligands tested (Fig. 3A) bound with significantly higher affinity to RG than to R (Fig. 4 and Table I). The extent of the preference for the RG complex over the R state varied from 12-fold for PTH(1–34) to 160-fold for PTHrP(1–36). In contrast, antagonist ligands did not appreciably discriminate RG from R (Fig. 4B and Table I); PTH(7–34) did not significantly discriminate RG from R, and PTH(3–34) bound with a slightly higher affinity (2-fold) to the uncoupled receptor than the RG complex (Table I). For displacement of $^{125}$I-PTH(3–34) binding, all ligands bound according to a single affinity state model, in agreement with the two-site model for the PTH1 receptor (Fig. 1, Equation 4). The $K_i$ values obtained provide a measure of the macro-affinity of the ligand for the R state of the receptor, a value incorporating both the affinity of the N interaction and of the J interaction ($1/(K_{N} + K_{J})$), see legend to Fig. 1 for explanation of model parameters.

For displacement of $^{125}$I-[MAP]PTHrP(1–36) binding, a single affinity state model described the binding of PTH(1–34) (Fig. 4A) and $[^{125}$I]-[MAP]PTHrP(1–36) (Fig. 4E), whereas a two affinity state model provided the best fit for PTHrP(1–36) (Fig. 4C) and [MAP]PTHrP(1–36) (Fig. 4D). The higher affinity of the two states most likely represents the affinity for RG, since for [MAP]PTHrP(1–36) the affinity of this state (2.7 nM) is equivalent to that of $^{125}$I-[MAP]PTHrP(1–36) (1.9 nM), the binding of which is largely GTPγS-sensitive (Fig. 2A). Therefore $K_{i\text{RG}}$ (Table I) for displacement of $^{125}$I-[MAP]PTHrP(1–36) binding provides an approximate measurement of the macro-affinity of the ligand ($1/(K_{N} + K_{J})$) for the RG state. ($K_{i\text{RG}}$ is the affinity from the single state fit for PTH(1–34) and $[^{125}$I]-[MAP]PTHrP(1–36), and the high affinity state for PTHrP(1–36) and [MAP]PTHrP(1–36.).

The 1–2 Region of PTH(1–34) Is a Determinant of R/RG Selectivity at the PTH1 Receptor—By assuming that the two-site model (Fig. 1, Equation 4) is appropriate for analysis of these ligand binding data (see below), the considerations above indicate that the macro-affinity ($K_{i\text{RG}}$) for agonist ligands was higher at the RG complex than the R state. We next determined the extent to which this increase resulted from an increase of ligand affinity for the N domain ($K_{N}$) and/or for subsequent ligand interaction with the J domain ($K_{J}$) of the receptor. The role of the J interaction was investigated first by examining the effect of modifications in the N-terminal region of the ligand.

The contribution of the 1–2 region of PTH(1–34) on R/RG binding selectivity was examined by comparing the binding of PTH(1–34) and PTH(3–34) to the PTH1 receptor. PTH(1–34)}
bound with 11-fold higher affinity to RG (displacement of \(^{125}\text{I}-\text{MAP}\)PTHrP(1–36)) than to R (displacement of \(^{125}\text{I}-\text{PTH}(3–34)\) in the presence of GTP\(_{\text{S}}\) (closed symbols, R state), and against \(^{125}\text{I}-\text{MAP}\)PTHrP(1–36) in the absence of GTP\(_{\text{S}}\) (open symbols, RG state). A, \([\text{Nle}^8,18,\text{Tyr}^{34}]\)bPTH(1–34); B, \([\text{Nle}^8,18,\text{Tyr}^{34}]\)bPTH(3–34); C, \([\text{Tyr}^{36}]\)PTHrP(1–36); D, \([\text{MAP}^{22–31},\text{Tyr}^{36}]\)PTHrP(1–36); and E, \([\text{Ile}^5,\text{MAP}^{22–31},\text{Tyr}^{36}]\)PTHrP(1–36)). Data were fit to single and two affinity state models (Equations 1 and 2, respectively), and the best fit was determined using a partial F test. For PTH(1–34), PTH(3–34), and \([\text{Ile}^5,\text{MAP}]\)PTHrP(1–36), a single affinity state model provided the best fit for inhibition of both radioligands, the two-state fit providing no improvement (\(p > 0.05\)). Inhibition of \(^{125}\text{I}-\text{MAP}\)PTHrP(1–36) binding by PTHrP(1–36) and \([\text{MAP}]\)PTHrP(1–36) was better described by a two-state fit (\(p < 0.05\)), whereas a single state model adequately described inhibition of \(^{125}\text{I}-\text{PTH}(3–34)\) binding by these two ligands (\(p > 0.05\)). Data were normalized as the percent of specific binding in the absence of unlabeled ligand. With one exception, the value of nonspecific binding used for normalizing the data was the fitted lower plateau of the displacement curve, which was in good agreement with the measured value of nonspecific binding (determined in the presence of 1.00 \(\mu\)M [MAP]PTHrP(1–36) or 300 nM [Nle\(^8,18,\text{Tyr}^{34}]\)bPTH(3–34)). The exception was inhibition of \(^{125}\text{I}-\text{PTH}(3–34)\) binding by [MAP]PTHrP(1–36), for which the measured value of nonspecific binding was used for normalizing the data and to define the lower plateau of the displacement curve in the curve-fitting analysis. Data are from representative experiments that were performed three times with similar results.

**Residue 5 of [MAP]PTHrP(1–36) Is a Determinant of R/RG Selectivity and a Determinant of Binding Affinity at the PTH1 Receptor**—We examined further the role of the N-terminal region of the ligand on R/RG selectivity by modification of residue 5 in [MAP]PTHrP(1–36). Residue 5 has previously been implicated in specifying the binding affinity of ligands for PTH receptors. Residue 5 controls the signaling selectivity of PTHrP for the PTH1 receptor over the PTH2 receptor. (Substitution of His\(^5\) in PTHrP by the equivalent residue in PTH(Ile) enables PTHrP to activate the PTH2 receptor (50, 51).) The same substitution increases the affinity of PTHrP for the PTH1 receptor (50, 51). In this study, His\(^5\) in [MAP]PTHrP(1–36) was replaced with Ile. The substitution modified the R/RG selectivity; [MAP]PTHrP(1–36) bound with 97-fold higher affinity to RG than to R (Fig. 4D and Table I). The selectivity was reduced to 17-fold for [Ile\(^5,\text{MAP}^{22–31},\text{Tyr}^{36}]\)PTHrP(1–36) (Fig. 4E and Table I). Position 5 is therefore a determinant of R/RG selectivity for [MAP]PTHrP(1–36).

The replacement of His\(^5\) by Ile also greatly increased the affinity of [MAP]PTHrP(1–36) for the uncoupled receptor (by 160-fold (Fig. 4, D and E, and Table I)), a larger effect than that previously observed for PTHrP (7-fold (50, 51)). The molecular basis of this affinity-enhancing effect is not known. We investigated the extent to which this effect was preserved at a
G-protein Regulation of the PTH1 Receptor

TABLE I
Ligand binding to the human PTH1 receptor, measured by inhibition of $^{125}$I-[MAP]PTHrP(1–36) binding, and by inhibition of $^{125}$I-PTH(3–34) binding in the presence of GTP-$\gamma$S

| Ligand                  | $^{125}$I-[MAP]PTHrP(1–36) | $^{125}$I-PTH(3–34) |
|-------------------------|-----------------------------|---------------------|
|                         | $p_K_{\text{high}}$ (GTP-$\gamma$S) | $p_K_{\text{low}}$ (GTP-$\gamma$S) | $K_{\text{GTPS}}$ (GTP-$\gamma$S) |
| [Nle$^8$,Tyr$^{34}$]bPTH(1–34) | 9.59 ± 0.05 (0.26) | 100 NA | 0 | 8.56 ± 0.04 (2.8) | 12 ± 2 |
| [Nle$^8$,Tyr$^{34}$]bPTH(3–34) | 8.83 ± 0.04 (2.4) | 100 NA | 0 | 8.91 ± 0.07 (1.2) | 0.53 ± 0.06 |
| [Nle$^{8,18}$,Tyr$^{34}$]bPTH(7–34) | 6.39 ± 0.11 (401) | 100 NA | 0 | 6.17 ± 0.10 (675) | 1.9 ± 0.06 |
| [MAP$^{22–31}$,Tyr$^{34}$]bPTHrP(1–36) | 9.50 ± 0.09 (0.31) | 83 ± 6 | 7.54 ± 0.16 (29) | 19 ± 7 | 7.35 ± 0.09 (44) | 160 ± 50 |
| [MAP$^{22–31}$,Tyr$^{34}$]bPTHrP(3–36) | 8.56 ± 0.07 (2.7) | 88 ± 8 | 6.20 ± 0.27 (626) | 12 ± 8 | 6.58 ± 0.10 (262) | 97 ± 7 |
| [Ile$^5$,MAP$^{22–31}$,Tyr$^{34}$]bPTHrP(1–36) | 10.0 ± 0.1 (0.067) | 100 NA | 0 | 8.81 ± 0.04 (1.6) | 17 ± 3 |
| [Ala$^{10,12}$,Arg$^{11}$]rPTH(1–14) | 6.27 ± 0.36 (540) | 26 ± 12 | 4.65 ± 0.13 (22,000) | 74 ± 12 | 4.95 ± 0.11 (11,000) | 30 |

a $p$ value is 0.0064.

\* $p$ value is 0.030.

\* $p$ value is 0.37.

\* $p$ value is 0.0063.

\* $p$ value is 0.00021.

\* $p$ value is 0.007.

\* $p$ value is 0.025.

\* Statistical significance was also tested for the difference between $p_K_{\text{GTPS}}$ and $p_K_{\text{low}}$ as 0.28.

\* Statistical significance was also tested for the difference between $p_K_{\text{GTPS}}$ and $p_K_{\text{low}}$ as 0.21.

\* Data were analyzed using an equation that assumes simultaneous binding of [Ala$^{10,12}$,Arg$^{11}$]rPTH(1–14) and $^{125}$I-PTH(3–34) (Equation 3, fitted parameters in legend to Fig. 5).

Inhibition of radioligand binding to the PTH1 receptor in HEK293 cell membranes was measured as described under “Experimental Procedures.” Data were fitted to equations assuming inhibition of radioligand binding to one (Equation 1) or two affinity states (Equation 2), and the best fit was determined using a partial $F$ test. Displacement of $^{125}$I-[MAP]PTHrP(1–36) binding provides a measurement of ligand binding to the G-protein-coupled state of the PTH1 receptor, whereas inhibition of $^{125}$I-bPTH(3–34) binding in the presence of GTP-$\gamma$S provides a measurement of ligand binding affinity for the uncoupled receptor. Statistical significance of the difference between $p_K_{\text{GTPS}}$ and $p_K_{\text{low}}$ was tested by Student’s $t$ test. NA, not applicable.

The ligand modification studies above indicate the direct effects of residue 5 on ligand interaction with the J domain of the receptor.

Detection of R/RG Selectivity of PTH(1–14) Binding to the PTH1 Receptor—The ligand modification studies above indicate that residues in the N-terminal region of the ligand (residues 1–2 and 5) are determinants of R/RG selectivity at the PTH1 receptor. These findings implicate ligand interaction with the J domain of the receptor in the preference for agonist ligands for the RG state. This hypothesis was examined more directly by measuring the R/RG selectivity of a ligand that interacts predominantly with the J domain, [Ala$^{10,12}$,Arg$^{11}$]rPTH(1–14) (PTH(1–14)). This N-terminal fragment activates the PTH1NT and wild-type receptors with equivalent potency (21). The ligand is a full agonist for stimulation of cAMP accumulation in COS-7 cells transfected with the PTH1NT receptor (Fig. 3B). This finding indicates that the enhancing effect of the N-term domain of the receptor controls R/RG selectivity of agonist binding to the PTH1 receptor.

The affinity state model. The high affinity state (540 nM) represented the RG state by examining the effect of overexpression of the G$_\alpha$($\alpha_{i}\beta_j$) mutant G-protein in COS-7 cells. A high affinity state for PTH(1–14) was not detected in membranes prepared from COS-7 cells expressing the PTH1 receptor alone (Fig. 5C). The lack of detectable high affinity binding in COS-7 cells is probably a result of the very high level of receptor expression in these cells, 1–2 million receptors/cell compared with 100,000 receptors/cell for the PTH1 receptor stably expressed in HEK293 cells.) Coexpression of the PTH1 receptor with G$_\alpha$($\alpha_{i}\beta_j$) produced a high affinity state of binding for PTH(1–14) ($K_{\text{high}}$ = 300 nM, representing 40 ± 9% of $^{125}$I-[MAP]PTHrP(1–36) binding displaced. Fig. 5C), demonstrating that the high affinity state is the RG complex. The detection of high affinity binding to the RG state for PTH(1–14) supports the hypothesis that ligand interaction with the J domain of the receptor controls R/RG selectivity of agonist binding to the PTH1 receptor.

$^{125}$I-PTH(3–34) Binds Predominantly to the N Domain of the Receptor and Does Not Appreciably Discriminate R and RG States of the PTH1 Receptor—A direct method with which to address the R/RG selectivity of the N interaction would be to measure R/RG selectivity of a ligand that only interacts with the N domain of the receptor. PTH(3–34) is a candidate ligand; circumstantial evidence suggests that this ligand may bind predominantly to the N domain. This domain of the receptor is a determinant of the selectivity of N-terminally truncated analogues of PTH(1–34) for the human PTH1 receptor over the rat PTH1 receptor (13). Dissociation of $^{125}$I-PTH(3–34) from the receptor from which most of the N-terminal domain had been removed (PTH1NT, residues 24–181 removed (21)). [MAP]PTHrP(1–36) failed to stimulate cAMP accumulation in COS-7 cells transfected with the PTH1NT receptor (Fig. 3B). Substitution of His$^8$ with Ile restored the ability of the ligand to stimulate cAMP accumulation via the PTH1NT receptor (Fig. 3B). This finding indicates that the enhancing effect of the substitution is preserved at a receptor from which the N domain has been largely removed, suggesting direct and/or indirect effects of residue 5 on ligand interaction with the J domain of the receptor.
G-protein Regulation of the PTH1 Receptor

PTH1 receptor is mono-exponential, consistent with a single site interaction (in contrast to the complex dissociation of $^{125}$I-PTH(1–34) which suggests more than one site of receptor-ligand interaction (48)). In this study we determined the extent to which PTH(3–34) binds to the J domain in a direct and quantitative fashion, by measuring the effect of blocking this domain on the binding affinity of the ligand. For this purpose we used [Ala$^{3,10,12}$,Arg$^{11}$]rPTH(1–14) (PTH(1–14), which binds predominantly if not exclusively to the J domain (see above).

The effect of PTH(1–14) on PTH(3–34) binding to the R state of the PTH1 receptor was measured by displacement of $^{125}$I-PTH(3–34) binding in the presence of 30 μM GTP·y·S, PTH(1–14) only partially inhibits the binding of the radioligand (Fig. 5A); analysis of the displacement data with a single affinity state model (Equation 1) indicated that PTH(1–14), at saturating concentrations, inhibited less than 5% of specific $^{125}$I-PTH(3–34) binding to the R state. This suggests that $^{125}$I-PTH(3–34) and PTH(1–14) can bind simultaneously to the receptor, since $^{125}$I-PTH(3–34) can still bind when the receptor is saturated with PTH(1–14). This in turn suggests that the binding sites on

**Fig. 5.** Inhibition of $^{125}$I-PTH(3–34) and $^{125}$I-[MAP]PTHrP(1–36) binding to the PTH1 receptor by PTH(1–14). A, displacement of $^{125}$I-PTH(3–34) binding to the PTH1 receptor in HEK293 cell membranes by [Ala$^{3,10,12}$,Arg$^{11}$]rPTH(1–14) in the absence (○) and presence (●) of GTP·y·S. Note that the PTH(1–14) analogue only partially inhibits binding of $^{125}$I-PTH(3–34). The curves are the best fits to an allosteric binding model (Equation 3) that allows for simultaneous binding to the receptor of both $^{125}$I-PTH(3–34) and PTH(1–14). The best fit parameters (mean ± S.E. from analysis of 3 or 4 independent experiments) were as follows: 30 μM GTP·y·S, $K_{(1–14)} = 96,000 ± 26,000 M^{-1}$ (10.4 μM), $a = 0.81 ± 0.06$; no GTP·y·S, $K_{(1–14)} = 71,000 ± 33,000 M^{-1}$ (14 μM), $a = 0.63 ± 0.03$. The α values are significantly different ($p = 0.025$). $K_{(3–34)}$ was held constant in the analysis at the value determined from the $^{125}$I-PTH(3–34) saturation experiments (3.2 × 10$^{-6}$ M, Fig. 2B). The experiment was performed three times for the presence of GTP·y·S and four times for the absence of the nucleotide, with similar results. B, displacement of $^{125}$I-[MAP]PTHrP(1–36) binding to the PTH1 receptor in HEK293 cell membranes by PTH(1–14). The curve is a two affinity state fit that provided a better fit than a single affinity state model ($p = 0.0045$). In this experiment the high affinity state (IC$_{50}$ of 250 nM) represents 13% of the total displacement of $^{125}$I-[MAP]PTHrP(1–36) binding. Data are from a single representative experiment that was performed three times with similar results.

**Fig. 6.** Inhibition of PTH(1–14)- and PTH(1–34)-stimulated cAMP accumulation in HEK293 cells expressing the PTH1 receptor by antagonist ligands. Antagonism of cAMP accumulation was measured as described under “Experimental Procedures” for inhibition of the effect of 10 μM [Ala$^{3,10,12}$,Arg$^{11}$]rPTH(1–14) (A) and 2 nM hPTH(1–34) (B). The following antagonists were tested: [Ne$^{6,10}$,Tyr$^{24}$]hPTH(3–34), [o-Trp$^{22}$,Tyr$^{24}$]hPTH(7–34), bTIP(7–39), and bTip39. Both the agonist and antagonist were added simultaneously, following a 30-min preincubation with assay buffer containing a phosphodiesterase inhibitor (Ro 20-1724). The assay was terminated after a 30-min incubation with the ligands. The data were analyzed using a four-parameter logistic equation to obtain estimates of IC$_{50}$, pseudo-Hill slope, total cAMP produced in the presence of the agonist but in the absence of antagonist (max), and cAMP produced in the presence of the agonist and a saturating concentration of the antagonist (min). For presentation purposes the data have been normalized as follows: % response = ($y$ – basal)$/(max – basal)$ × 100, where $y$ is the cAMP produced at a given concentration of antagonist and basal is the amount of cAMP produced in the absence of any ligand. Note that the ligands only partially antagonize the PTH(1–14) response (min is greater than basal). Data points are the mean ± range of duplicate measurements. Data are from representative experiments that were performed three times with similar results.
the receptor for $^{125}$I-PTH(3–34) and PTH(1–14) are, at least to an extent, spatially distinct, a finding that is fully consistent with the two-site model which assumes different binding sites on the receptor for N- and C-terminal portions of the ligand (12).

The PTH(1–14) versus $^{125}$I-PTH(3–34) displacement data were re-analyzed using a model that assumes the following: 1) binding of PTH(1–14) to the receptor and to the $^{125}$I-PTH(3–34)-occupied receptor, and 2) binding of $^{125}$I-PTH(3–34) to the receptor and to the PTH(1–14)-occupied receptor (Equation 3). The fitted values are given in the legend to Fig. 5. The affinity of $^{125}$I-PTH(3–34) for PTH(1–14)-occupied receptor was only slightly lower than the affinity of $^{125}$I-PTH(3–34) for the non-occupied receptor (3.9 and 3.1 nM, respectively). Occupancy of the J domain by PTH(1–14) therefore minimally affects the binding affinity of $^{125}$I-PTH(3–34) for the PTH1 receptor, strongly suggesting that almost all the binding energy of this ligand is supplied by the N interaction (Fig. 1). PTH(3–34) does not appreciably discriminate the R and RG states of the receptor (Fig. 4B), indicating that the N interaction is insensitive to receptor-G-protein coupling.

Validation of the Simultaneous Binding Model for PTH(1–14) and PTH(3–34) Interaction with the PTH1 Receptor—Simultaneous binding of PTH(3–34) and PTH(1–14) to the PTH1 receptor is suggested by the binding of $^{125}$I-PTH(3–34) to the receptor saturated with PTH(1–14) (Fig. 5A). Since occupancy of the receptor by PTH(1–14) only partially reduces the binding of PTH(3–34), this binding model predicts that occupancy of the receptor by PTH(3–34) should only partially reduce the binding of PTH(1–14). We tested this prediction by measuring the effect of PTH(3–34) on PTH(1–14)-stimulated cAMP production in HEK293PTH1 cells.

PTH(3–34) inhibited 10 μM PTH(1–14)-stimulated cAMP accumulation at the PTH1 receptor in a concentration-dependent fashion. However, the antagonist only partially inhibited the response to PTH(1–14) (Fig. 6A). In the presence of saturating PTH(3–34) concentrations, the PTH(1–14) response was 39 ± 6% of the cAMP accumulation in the absence of antagonist. This finding validates the simultaneous binding model for PTH(1–14) and PTH(3–34) interaction with the receptor. A variety of other PTH1 receptor antagonists were tested for their effect on PTH(1–14) signaling, using ligands varying in the number of residues truncated from the N terminus. All antagonist ligands tested only partially inhibited PTH(1–14)-stimulated cAMP accumulation (Fig. 6B). The magnitude of the PTH(1–14) response remaining in the presence of saturating concentrations of ligand was not significantly different for the different antagonists (p = 0.56 (single-factor analysis of variance), [o-Trp$^{12}$,Tyr$^{34}$]bPTH(7–34), 31 ± 6% of the PTH(1–14) response in the absence of antagonist; bTIP$^{39}$, 31 ± 1%; bTIP (7–39), 38 ± 5%).

The validity of the partial antagonism of the response of PTH(1–14) by these ligands was evaluated by testing the effect under conditions in which the antagonist ligands would be expected to inhibit completely the agonist-stimulated cAMP accumulation. We examined the antagonist effect on the stimulation of hPTH(1–34) of cAMP accumulation; the N interaction contributes the bulk of the binding energy for PTH(1–34)
of the PTH1 Receptor—The observation of higher affinity binding of agonist ligands to the RG versus the R state of the receptor implies a different conformation of the receptor when coupled to G-protein. Other ligand binding data in this study provide circumstantial evidence for a model of how the conformation of the receptor differs between the R and RG states. At the uncoupled receptor, PTH(1–14) and PTH(3–34) bind almost independently of each other (Fig. 5A), suggesting that the receptor conformation is “open” enough to allow access of both ligands to their binding sites on the receptor. PTH(1–14) more effectively blocks 125I-PTH(3–34) binding in the absence versus the presence of GTPγS; the negative cooperativity between the binding of 125I-PTH(3–34) and PTH(1–14) is significantly greater in the absence of GTPγS (Fig. 5A). Although this difference is small, only a small fraction of the receptor population is coupled to G-protein (22%), suggesting a considerable reduction of PTH(3–34) binding affinity resulting from PTH(1–14) occupancy at the RG state. This suggests that the conformation of the receptor is more “closed,” preventing simultaneous access of both ligands to their binding sites on the receptor. Further circumstantial evidence for a more closed conformation at the RG state is provided by the observation of pseudo-irreversible binding of agonist radioligands to the RG state (Fig. 2C (48)), suggesting that the ligand is trapped within the RG complex.

We tested this hypothesis more directly by increasing the fraction of PTH1 receptors in the RG state and by testing the effect of PTH(1–14) on the binding of 125I-PTH(3–34). COS-7 cells were transfected with either the PTH1 receptor alone or cotransfected with the PTH1 receptor and the mutant G-protein Gαs(α1β2). Coexpression with G-protein substantially increased the fraction of PTH1 receptors in the RG state. In membranes prepared from COS-7 cells expressing the PTH1 receptor alone, a high affinity state for [MAP]PTHrP(1–36) could not be detected in a 125I-PTH(3–34) displacement assay (Fig. 8A). In membranes containing the receptor and Gαs(α1β2), the high affinity state (IC50 = 270 pM) represented 40% ± 4% of the 125I-PTH(3–34) binding displaced by [MAP]PTHrP(1–36) (Fig. 8A). The additional high affinity binding produced by expression of the mutant G-protein was not entirely sensitive to GTPγS; the nucleotide did not completely remove the high

Assessment of Ligand Conformation at the R and RG States

(demonstrated by the >1000-fold lower potency of the ligand at the N-terminally truncated PTH1NT receptor compared with the wild-type receptor (21)). As a result ligands that are proposed to interact predominantly with the N domain, such as PTH(3–34), should effectively and completely antagonize the response to PTH(1–34). The data are in good agreement with this prediction—PTH(3–34), and the other antagonist ligands completely inhibited the response to 2 nM PTH(1–34) at the PTH1 receptor (Fig. 6B; PTH(3–34), 0.8 ± 7% of the PTH(1–34) response remaining; [n-Trp]125I-PTH(7–34), 3 ± 1%; bTIP39, 3 ± 1%; bTIP7(7–39), 1 ± 1%).

We examined the mechanism of the PTH(3–34)/s partial antagonism of PTH(1–14)/s stimulation of cAMP accumulation. The concentration dependence of PTH(1–14) for stimulation of cAMP production was measured in the presence of varying concentrations of PTH(3–34) (Fig. 7A), to determine the extent to which the antagonist affected the EC50 and/or Emax of PTH(1–14). PTH(3–34) reduced the Emax for PTH(1–14) (Fig. 7D). The antagonist also produced an increase of EC50 for PTH(1–14), but the magnitude of this increase reached a limiting value (18-fold), producing a hyperbolic Schild plot (Fig. 7C). Both the reduction of Emax and hyperbolic Schild plot can be explained by noncompetitive inhibition of the effect of PTH(1–14) by PTH(3–34), fully consistent with the simultaneous binding model for interaction of these two ligands with the PTH1 receptor. The hyperbolic Schild plot indicates that saturation of the receptor with PTH(3–34) only partially inhibits PTH(1–14)/s binding to and activation of the receptor (52), consistent with the ability of PTH(1–14) to bind the PTH(3–34)-occupied receptor. The reduction of Emax (Fig. 7D) indicates that binding of PTH(3–34) reduces the strength of signaling of the PTH(1–14)-occupied receptor (52). Unfortunately it is not possible to quantify the effect of PTH(3–34) on the binding affinity of PTH(1–14) without knowing the magnitude of receptor reserve for the agonist ligand (52). As anticipated (see above), PTH(3–34) behaved as a competitive antagonist for inhibition of PTH(1–34)/s stimulation of cAMP accumulation (Fig. 7B). PTH(3–34) did not affect Emax (Fig. 7D) and produced a linear increase of log(dose ratio −1) with a Schild slope of 1.14 ± 0.17 (Fig. 7C).

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affinity state (Fig. 8A). (The mechanism underlying this effect, which has also been observed for the β3-adrenergic receptor (43), is not presently understood.) PTH(1–14) did not appreciably inhibit binding of 125I-PTH(3–34) to the PTH1 receptor expressed alone; significant inhibition was observed at the highest concentration tested (100 μM), but we were unable to reliably fit the data to a single affinity state binding model (Equation 1) or the simultaneous binding model (Equation 3).

In contrast, PTH(1–14) inhibited 125I-PTH(3–34) binding to membranes with the receptor and Go1(αs/βs), with a maximal inhibition of 48% (Fig. 5B) and with an affinity (190 nM) similar to that for the RG state measured in competition against 125I-[MAP]PTHrP(1–36) (300 nM, Fig. 5C). Further evidence that this high affinity state represented the RG state was its removal by GTPγS (Fig. 8B). These data suggest that PTH(1–14) almost or completely inhibits the binding of 125I-PTH(3–34) to the 40% of the receptor population in the RG state. This finding is in good agreement with the hypothesis of a more closed conformation of the RG state that prevents simultaneous binding of PTH(1–14) and PTH(3–34).

**DISCUSSION**

Previous studies employing modified receptors and ligands have indicated a two-site model of ligand recognition by the PTH1 receptor. The C-terminal portion of the 1–34 fragment of PTH or PTHrP interacts with the extracellular N-terminal receptor region (N interaction), and the N-terminal portion of the ligand binds to the juxtamembrane domain of the receptor (J interaction) (11, 12). In this study we examined the effect of different receptor conformational states arising from receptor-G-protein interaction on the molecular mechanism of ligand binding to the PTH1 receptor. The principle findings of this study are as follows. 1) Receptor-ligand interaction for the unmodified receptor is well described by the two-site model. Strong, direct evidence for this binding mode was provided by the novel observation of allosteric interactions between the binding of PTH(1–14) and PTH(3–34) to the receptor together with the inference of simultaneous receptor binding of these two ligands. 2) Agonist ligands bind with higher affinity to the RG state than to the uncoupled receptor, whereas antagonist ligands bind with similar affinity to these states. 3) The J interaction is stabilized by R-G coupling, whereas the N interaction is not appreciably affected by R-G interaction. 4) A more closed receptor conformation is suggested for the PTH1 receptor when coupled to G-protein. These findings are summarized in the model in Fig. 9.

The observation of allosteric interactions between the binding of PTH(1–14) and PTH(3–34) confirms the two-site model for the unmodified PTH1 receptor. The inference of simultaneous binding of PTH(1–14) and PTH(3–34) is consistent with spatial and functional independence of receptor-binding sites that interact with the 1–14 and 15–34 regions of the ligand, as proposed previously (12). The allosteric analysis also provided insight into the mechanism of PTH(3–34) binding. PTH(1–14), used to block interactions of PTH(3–34) with the juxtamembrane domain (21), had very little effect on the receptor binding affinity of 125I-PTH(3–34) (1.25-fold increase of Kᵣ at the G-protein-uncoupled receptor). This finding indicates that PTH(3–34) does not interact appreciably with the juxtamembrane domain, implying that almost all the binding energy for PTH(3–34) is provided by the N interaction.

The effect of R-G-coupling on ligand binding to the receptor was evaluated by comparing ligand affinity for the uncoupled receptor with the affinity for the RG complex. The former was measured by displacement of 125I-PTH(3–34) binding in the presence of 30 μM GTPγS. For the latter we developed a new radioligand that selectively labels the RG state, 125I-[MAP]-

![Fig. 9. Model for modulation of ligand binding to the PTH1 receptor by G-protein.](Image)

**Fig. 9. Model for modulation of ligand binding to the PTH1 receptor by G-protein.** A, the C-terminal portion of the ligand interacts with the N domain of the receptor. Subsequently, the N-terminal portion of the ligand binds to the J domain of the receptor (B). C, R-G interaction increases the affinity of the J interaction, possibly by producing a closure of the receptor conformation. Reciprocally, interaction of the ligand with the J domain increases the affinity of receptor for G-protein, stimulating G-protein activation. Binding of G-protein to the other states of the receptor (R and RL) has been omitted for clarity.
and not through ligand binding to the N domain ($K_V$) (Fig. 9).

An increase of ligand affinity for RG compared with R indicates different receptor conformations at the G-protein-coupled and -uncoupled receptor states. At the uncoupled receptor, the allosteric binding data suggest that the conformation is open enough to permit the simultaneous binding of PTH(1–14) and PTH(3–34). For the RG state, three observations are consistent with the hypothesis of a more closed receptor conformation. 1) Agonist binding is pseudo-irreversible, suggesting that the ligand is trapped within the ligand-receptor-G-protein complex (48). 2) PTH(1–14) produces a greater reduction of the binding affinity of PTH(3–34) at the RG state, suggesting that simultaneous binding of one ligand better occludes binding of the second. 3) PTH(3–34) reduces the $E_{\text{max}}$ of PTH(1–14)-stimulated cAMP accumulation. This suggests a reduced ability of the PTH1 receptor to adopt an active conformation that couples to G-proteins when both PTH(3–34) and PTH(1–14) are bound to the receptor. The terms open and closed are used operationally. We offer no structural interpretation of the postulated open and closed states, since such an interpretation is beyond the scope of the present data. The hypothesis, currently based on indirect measurements of receptor conformation (ligand binding data), requires more direct examination.

Whereas this study has focused on the conformation of the receptor, the conformation of the ligand when bound to the receptor is largely unknown. For the free peptide, a recent receptor, the conformation of the ligand when bound to the receptor is largely unknown, and not through ligand binding to the N domain (ligand binding data), requires more direct examination.

In conclusion, for the first time we have extended the two-site model to take into account different conformations of the PTH1 receptor, the R and RG states. Agonist ligand interaction with the J domain of the PTH1 receptor discriminates the RG state from the R state suggesting that the J interaction increases receptor-G-protein interaction and enhances subsequent second messenger generation. Ligand binding to the N domain is insensitive to R-G interaction. Finally, the increase of agonist affinity for the RG state may result from a "closure" of the receptor conformation. Given the commonality of the low resolution binding mechanism for type IIGPCRs, these findings may well be relevant to an understanding of the signal transduction mechanism of other members of this receptor family.

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