Regions in Rat and Human Parathyroid Hormone (PTH) 2 Receptors Controlling Receptor Interaction with PTH and with Antagonist Ligands

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ABSTRACT

The parathyroid hormone (PTH) 2 receptor is potently activated by tuberoinfundibular peptide (TIP39). Rat and human PTH2 receptors differ considerably in their PTH responsiveness. PTH weakly stimulates cAMP accumulation via the rat receptor, and here we show it did not detectably increase intracellular calcium ([Ca\(^{2+}\)]) and bound with low affinity (450 nM). For the human PTH2 receptor PTH was a full agonist for increasing cAMP, a partial agonist for increasing [Ca\(^{2+}\)], and bound with high affinity (18 nM). In addition, the antagonists PTH(7–34) and TIP(7–39) bound with 10- to 49-fold lower affinity to the rat receptor. We investigated the molecular basis of differential PTH and antagonist interaction with human and rat PTH2 receptors by using chimeric human/rat PTH2 receptors. PTH cAMP-signaling efficacy (E_{\text{max}}) was determined by extracellular loop (EL) 1 and a region including EL2 and EL3. The N-terminal domain determined PTH binding selectivity at the inactive receptor state. Multiple regions throughout the receptor are required for the PTH-PTH2 receptor complex to adopt a high-affinity active state: inserting the rat receptor’s N-terminal domain, EL1 or EL2/3, into the human receptor increased PTH’s EC_{50} and reciprocal exchanges did not reduce EC_{50}. This suggests the global receptor conformation prevents the rat receptor from adopting a high-affinity state when in complex with PTH. N-terminal ligand truncation, producing the antagonists PTH(7–34) and TIP(7–39), altered ligand interaction with the membrane-embedded domain of the receptor, eliminating EL2/3 as a specificity determinant and lowering binding affinity. These insights should contribute to the development of a high-affinity PTH2 receptor antagonist, for investigating the receptor’s physiological role.

The parathyroid hormone (PTH) 2 receptor from various species (human, rat, and zebrafish) is potently activated by a recently identified neuropeptide, tuberoinfundibular peptide of 39 residues (TIP39) (Usdin et al., 1999; Hoare et al., 2000b). The physiological role of this new peptide-receptor system is currently being investigated. The distribution of the PTH2 receptor in spinal cord and its presence in dorsal root ganglion neurons suggests a role in pain perception (Usdin et al., 1999; Wang et al., 2000). The receptor is abundantly expressed in the median eminence and periventricular nucleus of the hypothalamus, suggesting PTH2 receptor involvement in the regulation of pituitary function (Usdin et al., 1999; Wang et al., 2000). The receptor belongs to the type II family of G protein-coupled receptors, which respond to peptide modulators such as secretin, glucagon, calcitonin, vasoactive intestinal polypeptide, and corticotropin-releasing hormone.

The PTH2 receptor and TIP39 form part of an extended family of related receptors and related ligands that also includes the PTH1 receptor, PTH, and PTH-related protein (PTHrP) (Hoare and Usdin, 2001). The ligands and receptors have presumably evolved to selectively mediate different physiological functions. In this regard, the PTH2 receptor is potently activated by TIP39 but not by PTHrP (Hoare and Usdin, 2001) and the PTH1 receptor is activated by PTH and PTHrP but not by TIP39 (Jüppner et al., 1991; Hoare and Usdin, 2001). The human PTH2 receptor was initially identified as a receptor for PTH based on potent PTH-stimulated cAMP accumulation via the receptor expressed in transfected cells (Usdin et al., 1995). In contrast the rat PTH2 receptor is poorly activated by PTH; the peptide is a low potency (20–100 nM) partial agonist for stimulation of cAMP production in transfected cells (Hoare et al., 1999; Usdin et al., 1999). The cAMP response to PTH is weak or undetectable for the rat PTH2 receptor expressed endogenously in F-11 cells, a dorsal root ganglion neuron-like cell line (Usdin et al., 1999, 2000b).

AABBREVIATIONS: PTH, parathyroid hormone; TIP39, tuberoinfundibular peptide; PTHrP, parathyroid hormone-related protein; [Ca\(^{2+}\)], intracellular calcium; N-domain, N-terminal extracellular receptor domain; J-domain, juxtamembrane receptor domain; b, bovine; h, human; m, mouse; r, rat; DMEM, Dulbecco’s modified Eagle’s medium; EL, extracellular loop; AEBSF, 4-[(2-aminoethyl)benzenesulfonyl]fluoride; RLU, relative light units; PLC, phospholipase C; HEK, human embryonic kidney; TM domain, transmembrane receptor domain.

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These findings suggest that PTH is not a physiologically significant activator of the PTH2 receptor in rats.

The human PTH2 receptor has been shown to couple to increases of intracellular calcium concentration ([Ca\(^{2+}\)]\text{i}) and inositol phosphates in response to PTH (Behar et al., 1996; Takasu et al., 1998) but the responsiveness of this signaling pathway to TIP39 has not been investigated. In addition, the possible coupling of the rat PTH2 receptor to increases of [Ca\(^{2+}\)]\text{i} has not been examined. This is important because the rat and human PTH2 receptors differ in their pharmacological profiles, and the investigation of the PTH2 receptor’s physiological roles is likely to be performed in rodents. The binding profile of the rat PTH2 receptor also has not been examined (prior to the isolation of TIP39, a radioligand has not been available for this receptor). It is not known whether the weak PTH activation of the rat PTH2 receptor is in part due to a low binding affinity. The first aim of this study was therefore to extend the comparison of human and rat PTH2 receptors by measuring the [Ca\(^{2+}\)]\text{i}, response and ligand binding affinity for PTH and TIP39.

Previous studies have used differences of ligand pharmacology between closely related receptors to investigate the functional role of molecular elements in ligand recognition and receptor activation (Holtmann et al., 1995; Stroop et al., 1995; Bergwitz et al., 1996, 1997; Couvineau et al., 1996; Turner et al., 1996, 1998; Clark et al., 1998; Dautzenberg et al., 1998; Hoare et al., 2000a). For the TIP39-PTH2 receptor interaction, the large extracellular N-terminal domain (N-domain) of the receptor is involved in TIP39 binding but not receptor activation. The membrane embedded “juxtamembrane” domain (J-domain) is a determinant of receptor activation by TIP39 and also contributes to its binding affinity (Hoare et al., 2000a). Previous studies have also identified regions and sequences within the human PTH2 receptor that limit its interaction with PTHrP (Bergwitz et al., 1997; Clark et al., 1998; Turner et al., 1998). However, the functional role of PTH2 receptor regions in PTH binding and signaling has not been investigated. We used chimeric human/rat PTH2 receptors to identify PTH2 receptor regions involved in PTH binding and signaling.

Moderate-affinity antagonists have been developed for the human PTH2 receptor by modification of the N-terminal region of PTH or TIP39 (Behar et al., 1996; Gardella et al., 1996; Hoare et al., 2000a) [e.g., PTH(7–34) and TIP(7–39)]. A high-affinity antagonist for the rat receptor would be very useful for investigating the physiological role of the PTH2 receptor. In this study we compared antagonist binding affinity for human and rat PTH2 receptors and found that the antagonists tested bound the rat receptor with low affinity. We used chimeric human/rat PTH2 receptors to identify the molecular basis of the weakened interaction of antagonist ligands with the rat PTH2 receptor. In addition, the receptor antagonists for agonist binding [PTH(1–34) and TIP39] were compared with those for the corresponding N-terminally truncated antagonists [PTH(7–34) and TIP(7–39)], to examine the possible changes of receptor-ligand interaction involved in the loss of activation produced by truncation of the ligand. These studies provided insight into the molecular basis of antagonism of rat and human PTH2 receptors, which should contribute to the development of a high-affinity antagonist for the rat PTH2 receptor.
ing protein aequorin). The following day, for cAMP and radioligand binding assays, cells were transferred after trypsinization to 96-well plates at a density of 50,000 cells/well.

**Measurement of Cellular Levels of cAMP.** After removal of medium, transfected COS-7 cells were treated at 37°C with 50 μl/well cAMP assay buffer (DMEM containing 25 mM HEPES supplemented with 0.1% bovine serum albumin, 30 μM Bt 20-1724 (Sigma/RBI, Natick, MA), 100 μM 4-2-aminoethylbenzenesulfonyl fluoride (AEBSF), and 1 μg/ml bacitracin). After 40 min this buffer was removed and replaced with 40 μl of the same supplemented buffer. Test agents were added in a volume of 10 μl and the cells incubated for an additional 40 min at 37°C. The assay was then terminated by the addition of 50 μl 0.1 N HCl, 0.1 mM CaCl₂. cAMP was quantified using a radioimmunoassay as previously described (Clark et al., 1998).

**Measurement of Intracellular Calcium.** Intracellular calcium was measured using aequorin luminescence (Button and Brownstein, 1993). Three days after transfection, COS-7 cells coexpressing aequorin and receptor cDNA were washed once with phosphate-buffered saline then loaded for 2 h with 2.5 μM coelenterazine hcp (Molecular Probes, Eugene, OR) in DMEM supplemented with 0.1% fetal bovine serum, 30 μM glutathione (reduced form) and 25 mM HEPES. Cells were then washed with Dulbecco's phosphate-buffered saline containing 1 mM Ca²⁺ and 1 mM Mg²⁺ supplemented with 1% bovine serum albumin (DBPBS buffer) and then dislodged in a 10-ml volume of the same buffer by gentle pipetting. The cell number was adjusted to 20,000 to 50,000 cells/ml and 0.2 ml cells added to 12 × 75-mm tubes. Test agents were made up in DBPBS buffer supplemented with 1 μg/ml bacitracin and 100 μM AEBSF. Baseline luminescence of the cells was measured for approximately 10 s in a EG&G Berthold Lumat LB9507 luminometer followed by manual addition of 50 μl test agent and immediate initiation of luminescence measurement. After a further 60 s (during which time luminescence for all the experimental conditions returned to baseline) 50 μl of 0.05% Triton X-100 was added, using an automatic injector, to permeabilize the cells. The remaining aequorin activity was measured for 50 s, during which time luminescence returned to baseline. Measurements of relative light units (RLU) were recorded every second. In each assay duplicate time courses were measured for each test agent. The calcium response was quantified as the percentage of total RLU, the summed RLU in response to ligand in 60 s divided by the total RLU (that produced by ligand added to that produced by saturation of aequorin with Ca²⁺ for 50 s after addition of Triton X-100). We initially attempted this assay with HEK293T cells but observed a substantial increase of [Ca²⁺]i, in nontransfected HEK293T cells in response to PTH and PTHrP but not TIP39, suggesting the presence of an endogenous PTH1 receptor.

**Whole-Cell Radioligand Binding Assays.** Binding of unlabeled ligands was measured by displacement of 125I-hTIP39 binding to PTH2 receptor-expressing COS-7 cells in 96-well plates. Cells were washed once with 100 μl of binding buffer (50 mM Tris, 100 mM NaCl, 5 mM KCl, 2 mM CaCl₂, pH 7.5 with HCl, supplemented with 5% heat-inactivated horse serum, 0.5% fetal bovine serum, 1 μg/ml bacitracin, and 100 μM AEBSF). To each well was added sequentially 65 μl of binding buffer, 10 μl of unlabeled ligand diluted in binding buffer, and 25 μl of radioligand diluted in binding buffer (approximately 50,000–100,000 cpm/well). Total binding was defined in the absence of unlabeled ligand and nonspecific binding was measured in the presence of 1 μM TIP39. Cells were incubated at 15°C for 3 h. The assay plates were placed on ice for 10 min and then washed twice with 100 μl/well binding buffer. Cell-associated radioactivity was extracted with 200 μl of 1.0 N NaOH. Samples were transferred to tubes and radioactivity measured in a Wallac 1470 Wizard gamma counter. Nonspecific binding was 3 to 5% of the total counts added. Total binding was less than 20% of added radioactivity.

**Data Analysis.** Concentration dependence data was analyzed using the following four-parameter logistic equation with Prism 2.01 (GraphPad Software, San Diego, CA):

\[
y = \min + \frac{\max - \min}{1 + 10^{X - X^*}}
\]

where \(X\) is the logarithm of the ligand concentration and \(n\) is Hill slope. For cAMP accumulation data \(y\) is the amount of cAMP accumulated at a given peptide concentration, \(min\) is the cAMP level in the absence of ligand, \(max\) is the maximum level produced, and \(K\) is the logEC₅₀. For intracellular calcium, \(y\) is the percentage of total RLU produced by a given ligand concentration, \(min\) is the percentage of total RLU produced in the absence of ligand, \(max\) is the maximal ligand-stimulated response, and \(K\) is the logEC₅₀. For inhibition of radioligand binding, \(y\) is the cpm bound at a given unlabeled ligand concentration.
Results

Comparison of Mouse and Human/Bovine TIP39. The sequence of bovine TIP39 has been previously described (Usdin et al., 1999). Database searching revealed that the amino acid sequence of human TIP39 (hTIP39) is identical to that of bovine TIP39 and that mouse TIP39 (mTIP39) differs at four positions in the carboxyl-terminal portion of the peptide. In mTIP39, Arg replaces His24 of the human/bovine sequence, Asp replaces Asn27, Gln replaces His31, and Leu replaces Val35. In cAMP accumulation assays mTIP39 was equivalently active to hTIP39 for both the human (Fig. 3A) and rat (Fig. 3B) PTH2 receptors (see legend to Fig. 3).

Coupling of Human and Rat PTH2 Receptors to Increases of Intracellular Calcium. TIP39 and PTH peptides produced a rapid, transient elevation of \([\text{Ca}^{2+}]_i\) in COS-7 cells expressing the human PTH2 receptor (Fig. 2A). hPTH(1–34) was a partial agonist compared with the maximal effect of hTIP39. hPTH(1–34) was also less potent, with a 5.8-fold higher EC\(_{50}\) compared with the EC\(_{50}\) of hTIP39 (3.8 nM) (Fig. 3C; Table 1). rPTH(1–34) was less potent (EC\(_{50}\) nM) (Fig. 3C; Table 1). rPTH(1–34) was a partial agonist compared with the maximal effect of hTIP39. hPTH(1–34) was also less potent, with a 5.8-fold higher EC\(_{50}\) compared with the EC\(_{50}\) of hTIP39 (3.8 nM) (Fig. 3C; Table 1). rPTH(1–34) was less potent (EC\(_{50}\) nM) (Fig. 3C; Table 1). rPTH(1–34) was a partial agonist compared with the maximal effect of hTIP39. hPTH(1–34) was also less potent, with a 5.8-fold higher EC\(_{50}\) compared with the EC\(_{50}\) of hTIP39 (3.8 nM) (Fig. 3C; Table 1). rPTH(1–34) was less potent (EC\(_{50}\) nM) (Fig. 3C; Table 1).

Fig. 2. Time course of intracellular calcium concentration in COS-7 cells expressing human and rat PTH2 receptors in response to TIP39 and PTH. Luminescence was measured as described under Materials and Methods for COS-7 cells coexpressing aequorin and the human PTH2 receptor (A), rat PTH2 receptor (B and C), or \(\beta\)-galactosidase (D). Luminescence (RLU) was recorded at 1-s intervals, except for a 2-s delay after the time of addition of ligand or buffer alone (indicated by the arrow). The PLC inhibitor U73122 was preincubated with the cells for 20 min at room temperature prior to addition of mTIP39. Pertussis toxin (PTX, 100 ng/ml) was incubated with the cells overnight prior to the assay. Data were normalized as the percentage of the total RLU (where total RLU is the summed RLU produced by the ligand added to the summed RLU produced by permeabilization of the cells with Triton X-100). Data points are the mean ± range of measurements from duplicate time courses. Data are from representative experiments that were performed two to five times with similar results.

Fig. 3. Agonist pharmacology of human and rat PTH2 receptors expressed in COS-7 cells. Agonist concentration dependence relationships were measured for stimulation of cAMP accumulation (A and B), increase intracellular calcium (C and D), and receptor binding (E and F). Data for the human PTH2 receptor are presented in A, C, and E, and the rat PTH2 receptor in B, D, and F. The assays were performed as described under Materials and Methods. A and B, cAMP was measured after a 40-min incubation with the ligands and the data expressed as a percentage of the maximal response to hTIP39, which was included as a reference at 1 \(\mu\)M concentration for each concentration dependence assay. The \(-\log EC_{50}\) and \(E_{\text{max}}\) values for hTIP39 and hPTH(1–34) are given in Table 3. For the human PTH2 receptor the \(-\log EC_{50}\) values for mTIP39 and rPTH(1–34) were, respectively, 9.32 ± 0.16 and 9.84 ± 0.08 with corresponding \(E_{\text{max}}\) values of 97 ± 8 and 118 ± 13%. For the rat PTH2 receptor the \(-\log EC_{50}\) values for mTIP39 and rPTH(1–34) were 9.29 ± 0.10 and 7.43 ± 0.24 with corresponding \(E_{\text{max}}\) values of 89 ± 6 and 58 ± 5%. C and D, \([\text{Ca}^{2+}]_i\) was measured as the summed RLU over a 60-s time course and the data normalized and analyzed as described under Materials and Methods. E and F, binding of unlabeled agonist ligands was measured by displacement of \(125\text{I}-\text{hTIP39}\) binding to COS-7 cells expressing the receptors. In all cases, the data points are the mean ± range of duplicate measurements. The curves are fits to a four-parameter logistic equation. Data are from representative experiments that were performed three to seven times with similar results.

equivalently active to hTIP39 (Fig. 3C; Table 1). PTH is therefore a weaker agonist than TIP39 for increasing \([\text{Ca}^{2+}]_i\), via the human PTH2 receptor. For the rat PTH2 receptor TIP39 (Fig. 2B) produced a rapid, transient increase of \([\text{Ca}^{2+}]_i\), similar to that observed for the human receptor (Fig. 2A). The concentration dependence relationship for hTIP39 and mTIP39 was again indistinguishable (Fig. 3D; Table 1). However, no detectable increase of \([\text{Ca}^{2+}]_i\) was observed for either rPTH(1–34) (Figs. 2B and 3D) or hPTH(1–34) (Fig. 3D) when tested at the high concentration of 10 \(\mu\)M. In addition, no increase was detectable for a lower concentration of rPTH(1–34) or hPTH(1–34) (100 nM) and for 320 nM hPTH(1–84) (data not shown). Therefore, PTH does not detectably affect \([\text{Ca}^{2+}]_i\), via the rat PTH2 receptor expressed in COS-7 cells. The mTIP39-stimulated increase of \([\text{Ca}^{2+}]_i\) was blocked by the phospholipase C (PLC) inhibitor U73122 (Fig. 2C), unaffected by the inactive analog U73343 (data not shown).
Pharmacological characterization of PTH2 receptor coupling to increases of intracellular calcium and cAMP in COS-7 cells

For both human and rat PTH2 receptors PTH displays a lower efficacy for increasing [Ca\(^{2+}\)], than for elevation of cAMP accumulation (Fig. 2C). The [Ca\(^{2+}\)], response to the PTH2 receptor therefore involves PLC and a calcium signaling pathway. It must reflect a difference in the signaling efficacy of PTH1 and PTH2 receptors (3.4- and 5.4-fold, respectively; Fig. 3, E and F; Table 2). For both human and rat PTH2 receptors mTIP39 bound with higher affinity to the human PTH2 receptor (6.2 nM), an 85- to 18-fold higher affinity than the other antagonists tested (Fig. 4A; Table 2). Previously it has been determined that truncation of six amino acid residues from hTIP39 eliminated detectable stimulation of the human PTH2 receptor (Hoare et al., 2000a). With the aim of improving antagonist affinity we tested mTIP7–39, because mTIP39 bound with higher affinity than hTIP39 (Fig. 3E; Table 2), mTIP7–39 bound with high affinity to the human PTH2 receptor (6.2 nM), an 8.5- to 18-fold higher affinity than the other antagonists tested (Fig. 4A; Table 2). mTIP7–39 did not produce a detectable increase of cAMP accumulation in COS-7 cells expressing the human PTH2 receptor [cAMP(basal) = 0.75 ±

<table>
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<tr>
<th>Peptide</th>
<th>hPTH2</th>
<th>rPTH2</th>
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<tr>
<td></td>
<td>[Ca(^{2+})], EC(_{50}) (nM)</td>
<td>[Ca(^{2+})], EC(_{50}) (nM)</td>
</tr>
<tr>
<td>hTIP39</td>
<td>8.42 ± 0.20 (3.8)</td>
<td>7.84 ± 0.40 (15)</td>
</tr>
<tr>
<td>mTIP39</td>
<td>8.76 ± 0.12 (1.8)</td>
<td>8.63 ± 0.20 (2.3)</td>
</tr>
<tr>
<td>hPTH(1–34)</td>
<td>7.65 ± 0.25 (22)</td>
<td>N.D.</td>
</tr>
<tr>
<td>rPTH(1–34)</td>
<td>7.78 ± 0.28 (16)</td>
<td>N.D.</td>
</tr>
<tr>
<td>PThrP(1–34)</td>
<td>5 ± 9*</td>
<td>12 ± 2*</td>
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</table>

N.D., not determined; N.A., not applicable.

More weakly coupled to increasing [Ca\(^{2+}\)], than to increasing cAMP accumulation.

Ligand Binding to Human and Rat PTH2 Receptors.
For both cAMP and [Ca\(^{2+}\)], signaling PTH activation of the rat PTH2 receptor is weaker than PTH activation of the human receptor. We measured receptor binding of the ligands, to determine the extent to which a difference of binding affinity between the receptors contributes to differential PTH responsiveness. In addition, several moderate-affinity antagonists have been described for the human PTH2 receptor. We measured their binding affinity for the rat receptor to assess their utility as antagonists for this receptor.

hTIP39 and mTIP39 displayed slightly higher affinity for displacement of \(^{125}\)I-hTIP39 binding to COS-7 cells expressing the human PTH2 receptor than to cells expressing the rat receptor (3.4- and 5.4-fold, respectively; Fig. 3, E and F; Table 2). For hPTH(1–34) and rPTH(1–34) the human PTH2 receptor selectivity was larger (31- and 53-fold, respectively). The affinity of hPTH(1–34) for the rat receptor was particularly low (450 nM) (Fig. 3F; Table 2). The rat PTH2 receptor therefore binds PTH with lower affinity than the human receptor. For both human and rat receptors mTIP39 bound with higher affinity than hTIP39 (17- and 12-fold higher, respectively).
Antagonist binding affinity for human and rat PTH2 receptors

Binding of unlabeled ligands to human and rat PTH2 receptors was measured by displacement of \(^{125}\text{I}-\text{TIP39}\) binding to intact COS-7 cells expressing the receptors. Data were analyzed using a four-parameter logistic equation as described under Materials and Methods. Data are the mean ± S.E.M. from three to seven experiments.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>hPTH2</th>
<th>rPTH2</th>
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<tr>
<td></td>
<td>(-\log IC_{50}) (IC(_{50}), nM)</td>
<td>(-\log IC_{50}) (IC(_{50}), nM)</td>
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<tr>
<td>Agonists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hTIP39</td>
<td>7.58 ± 0.06 (26)</td>
<td>7.04 ± 0.03 (91)</td>
</tr>
<tr>
<td>mTIP39</td>
<td>8.83 ± 0.14 (1.5)</td>
<td>8.12 ± 0.08 (7.5)</td>
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<tr>
<td>hPTH(1–34)</td>
<td>7.74 ± 0.17 (18)</td>
<td>6.34 ± 0.14 (450)</td>
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<tr>
<td>rPTH(1–34)</td>
<td>5.44 ± 0.10 (3.6)</td>
<td>6.79 ± 0.08 (160)</td>
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<tr>
<td>PTHrP(1–34)</td>
<td>6.07 ± 0.18 (850)</td>
<td>5.29 ± 0.04 (5,100)</td>
</tr>
<tr>
<td>Antagonists</td>
<td></td>
<td></td>
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<tr>
<td>hTIP(7–39)</td>
<td>7.18 ± 0.11 (67)</td>
<td>6.08 ± 0.05 (830)</td>
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<tr>
<td>mTIP(7–39)</td>
<td>8.21 ± 0.07 (6.2)</td>
<td>6.53 ± 0.02 (300)</td>
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<tr>
<td>[D-Tyrp12]bPTH(7–34)</td>
<td>7.28 ± 0.05 (53)</td>
<td>6.27 ± 0.02 (540)</td>
</tr>
<tr>
<td>PTHrP(1–21)/PTH(22–34)</td>
<td>6.96 ± 0.07 (110)</td>
<td>5.49 ± 0.01 (3,200)</td>
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</table>

Fig. 4. Antagonist binding to human and rat PTH2 receptors expressed in COS-7 cells. Binding of unlabeled antagonist ligands was measured by displacement of \(^{125}\text{I}-\text{TIP39}\) binding to intact COS-7 cells expressing the human PTH2 receptor (A) and the rat PTH2 receptor (B). Data points are the mean ± range of duplicate measurements. The curves are fitted to a four-parameter logistic equation. Data are from representative experiments that were performed three to five times with similar results.

0.33 pmol/well, cAMP(1 mM mTIP(7–39)) = 0.70 ± 0.15 pmol/well. Functional antagonism of mTIP(7–39) was demonstrated by a rightward shift of the mTIP39 concentration dependence curve for cAMP accumulation (data not shown), with a \(pK_b\) of 7.05 ± 0.21 (\(K_b\) = 88 nM). We prepared a \(^{125}\text{I}-\text{mTIP}(7–39)\) bound specifically to COS-7 cells expressing the human PTH2 receptor and also to membranes prepared from HEK293 cells stably expressing the receptor. [The signal-to-background ratios were 4:1 and 12:1, respectively.]

All of the antagonist ligands tested bound with much lower affinity to the rat PTH2 receptor than to the human receptor (10–49-fold lower; Fig. 4B; Table 2). For hTIP39 and mTIP39, removing residues 1 to 6 produces a larger reduction of affinity for the rat PTH2 receptor (9.1- and 40-fold reduction, respectively) than for the human PTH2 receptor (2.6- and 4.1-fold reduction, respectively). This finding suggests that the 1 to 6 portion of TIP39 contributes more binding energy for interaction with the rat receptor than for interaction with the human receptor. The weaker affinity of mTIP(7–39) for the rat receptor compared with the human receptor was confirmed by measuring its \(pK_b\) for antagonism of mTIP(7–39)-stimulated cAMP accumulation. The antagonist potency for the rat receptor (\(pK_b\) = 6.03 ± 0.19, \(K_b\) = 940 nM) was an order of magnitude lower than the potency for the human receptor (\(K_b\) = 88 nM).

Regions of Human and Rat PTH2 Receptors Specifying Differential PTH-Stimulated cAMP Production. PTH from various species is a low-potency partial agonist for the rat PTH2 receptor, relative to TIP39, but acts as a highly potent full agonist for the human receptor (for review, see Hoare and Usdin, 2001). Chimeric rat/human PTH2 receptors were used to identify regions in the PTH2 receptor that contributed to this differential responsiveness to PTH. The amino acid sequences of human and rat PTH2 receptors display considerable homology (82%; Fig. 1). Within the predicted transmembrane domains sequence identity is particularly high (95%) and the substitutions are nearly all conservative (Fig. 1). There are differences within the predicted extracellular regions: N-domain, EL1, EL2, and EL3. The large, 40-amino acid EL1 is particularly divergent, with only 65% sequence identity (Fig. 1). We constructed chimeric receptors in which extracellular regions were exchanged between the human and rat PTH2 receptors, because these regions are most likely involved in receptor-ligand interaction. Receptors were constructed that exchanged the N-domain (hN-rPTH2 and rN-hPTH2), a region from TM1 to TM3 conserved except for EL1 (hEL1-rPTH2 and rEL1-hPTH2), and a region from TM3 to the C terminus, including EL2 and EL3 (rN–TM3-hPTH2 and hN–TM3-rPTH2) (for systematic purposes the nomenclature describes the insertion of the
N-terminal-most receptor portion of one species homolog into the remainder of the other species homolog. For all the PTH2 receptors, chimeric and wild-type, the hTIP39 efficacy ($E_{\text{max}}$) was similar, suggesting a similar level of cell surface expression (Table 3; Fig. 5). The hTIP39 potency (EC$_{50}$) was also similar (Table 3; Fig. 5), suggesting that the conformation of the chimeric PTH2 receptors was not significantly disrupted.

We used human PTH(1–34) to examine PTH’s interaction with the chimeric receptors because among PTH analogs it displays the largest difference of $E_{\text{max}}$ and EC$_{50}$ between the rat and human PTH2 receptors (Hoare et al., 1999). The $E_{\text{max}}$ for human hPTH(1–34) was indistinguishable from that of hTIP39 at the human PTH2 receptor (99%; Fig. 5A; Table 3) but was only 32% of the maximal hTIP39 response at the rat PTH2 receptor (Fig. 5B; Table 3). Full agonism of hPTH(1–34) was between 0.3 and 1.0 pmol/well.

**TABLE 3**

**Stimulation of cAMP accumulation by TIP39 and PTH(1–34) at wild-type and chimeric human/rat PTH2 receptors**

Wild-type and chimeric PTH2 receptors were transiently expressed in COS-7 cells. Stimulation of cAMP accumulation was measured and the data analyzed as described under Materials and Methods. Values are the mean ± S.E.M. from three to seven experiments. The $E_{\text{max}}$ values for PTH(1–34) are agonist-specific cAMP accumulations. Basal cAMP was between 0.3 and 1.0 pmol/well.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$-\log$EC$<em>{50}$ (EC$</em>{50}$, nM)</th>
<th>$E_{\text{max}}$</th>
<th>$-\log$EC$<em>{50}$ (EC$</em>{50}$, nM)</th>
<th>$E_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPTH2</td>
<td>9.21 ± 0.34 (0.62)</td>
<td>2.6 ± 0.4</td>
<td>8.82 ± 0.24$^a$ (1.5)</td>
<td>99 ± 13$^b$</td>
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<tr>
<td>rPTH2</td>
<td>8.77 ± 0.34 (1.7)</td>
<td>2.9 ± 0.9</td>
<td>7.03 ± 0.15$^c$ (93)</td>
<td>32 ± 2$^d$</td>
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<td>hN-rPTH2</td>
<td>8.78 ± 0.54 (1.7)</td>
<td>3.4 ± 0.8</td>
<td>6.46 ± 0.76$^c$ (350)</td>
<td>26 ± 4$^e$</td>
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<tr>
<td>rN-hPTH2</td>
<td>8.81 ± 0.46 (1.5)</td>
<td>4.4 ± 1.0</td>
<td>7.17 ± 0.53$^c$ (68)</td>
<td>107 ± 4$^f$</td>
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<tr>
<td>hEL1-rPTH2</td>
<td>8.88 ± 0.34 (1.3)</td>
<td>2.9 ± 0.6</td>
<td>6.68 ± 0.30$^c$ (210)</td>
<td>64 ± 7$^{bc,d}$</td>
</tr>
<tr>
<td>rEL1-hPTH2</td>
<td>8.95 ± 0.20 (1.1)</td>
<td>3.5 ± 1.2</td>
<td>7.28 ± 0.14$^c$ (52)</td>
<td>83 ± 4$^b$</td>
</tr>
<tr>
<td>hN–TM3-rPTH2</td>
<td>8.68 ± 0.67 (2.1)</td>
<td>3.2 ± 1.2</td>
<td>7.04 ± 0.19$^c$ (91)</td>
<td>57 ± 7$^b$</td>
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<tr>
<td>rN–TM3-hPTH2</td>
<td>8.47 ± 0.36 (3.4)</td>
<td>4.1 ± 1.1</td>
<td>6.18 ± 0.46$^c$ (660)</td>
<td>56 ± 4$^{bc,d}$</td>
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For each ligand, statistical comparison of the data for the different receptors was performed using single-factor analysis of variance followed by post hoc analysis using the Newman-Keuls test. For TIP39 no significant difference was found between the $-\log$EC$_{50}$ values or between the $E_{\text{max}}$ values ($P = 0.99$ and 0.86, respectively). Significant difference was found between the $-\log$EC$_{50}$ values for PTH(1–34) ($P = 0.0002$): $-\log$EC$_{50}$ values marked $a$ are significantly different from the hPTH(1–34) value for the rat PTH2 receptor, and those marked $c$ are significantly different from the hPTH(1–34) value at the human PTH2 receptor. Significant difference was found between the PTH $E_{\text{max}}$ values ($P < 0.0001$): $E_{\text{max}}$ values marked $b$ are significantly different from the hPTH(1–34) value for the rat receptor and those marked $d$ are significantly different from the hPTH(1–34) value for the human receptor.
34) was observed with a chimeric receptor comprising the J-domain of the human PTH2 receptor and N-domain of the rat receptor (rN-hPTH2, 107% of the hTIP39 $E_{\text{max}}$; Fig. 5D). For the reciprocal receptor chimera (hN-rPTH2) the maximal effect of hPTH(1–34) (26%; Fig. 5C) was not significantly different from that for the rat PTH2 receptor (Table 3). The J-domain is therefore a determinant of hPTH(1–34)’s efficacy at the PTH2 receptor.

A partial restoration of the hPTH(1–34) $E_{\text{max}}$ was observed by exchange of smaller portions of the J-domain of the PTH2 receptor. Incorporating the carboxyl-terminal portion of the J-domain of the human receptor (rN–TM3-hPTH2) significantly increased the hPTH(1–34) $E_{\text{max}}$ (56%; Fig. 5H) compared with the wild-type rat PTH2 receptor (32%; Table 3). Reciprocally, transfer of the same region of the rat receptor into the human receptor (hN→TM3-rPTH2) significantly decreased the hPTH(1–34) response relative to the wild-type human PTH2 receptor (from 99 to 57%; Fig. 5G; Table 3). Incorporating EL1 of the J-domain of the human receptor into the rat receptor (hEL1-rPTH2) significantly increased the maximal effect of hPTH(1–34) to 64% (Fig. 5E). Transfer of the same region of the rat receptor to the human receptor (rEL1-hPTH2) appeared to lower the hPTH(1–34) $E_{\text{max}}$ (from 99 to 83%; Fig. 5F) but the difference was not statistically significant (Table 3). These findings indicate that multiple regions of the J-domain contribute to the differential hPTH(1–34) maximal responsiveness of the human and rat PTH2 receptors, including the divergent EL1 and a region including EL2 and EL3.

The potency of hPTH(1–34) for stimulation of cAMP accumulation was much lower at the rat PTH2 receptor (EC$_{50}$ = 93 nM) than at the human PTH2 receptor (1.5 nM). However, neither the N-domain nor the J-domain of the human PTH2 receptor restored high potency activation by hPTH(1–34) when transferred to the rat PTH2 receptor (Fig. 5; Table 3). Similarly high hPTH(1–34) potency was not restored by combination of the N-domain of the human PTH2 receptor with either the human EL1 or the human C-terminal portion of the J-domain (Fig. 5, G and F; Table 3). This finding suggests that molecular elements in the N-domain and in multiple regions of the J-domain are required in combination to determine the higher potency of hPTH(1–34) for the human PTH2 receptor.

Regions of Human and Rat PTH2 Receptors Specifying Differential hPTH(1–34) and hTIP39 Binding.

hPTH(1–34) binds with considerably lower affinity to the rat PTH2 receptor (450 nM) than the human receptor (18 nM; Table 2). It was also found that hTIP39 bound with slightly lower affinity to the rat receptor (3.4-fold; Table 2). In the whole-cell binding assay used, ligand binding to the high-affinity active state of the receptor is unlikely to contribute significantly to the specific binding signal. Due to the presence of high intracellular concentrations of guanine nucleotides, which break down the receptor-G protein complex, this state probably represents a transient intermediate (Gilman, 1987). We have argued that the receptor states identified in this assay are the G protein-uncoupled receptor or desensitized or internalized receptor states (Hoare and Usdin, 2001). We investigated the molecular determinants of ligand binding to these inactive states of the PTH2 receptor by using this assay. [We previously measured ligand affinity for defined active (G protein-coupled) and inactive receptor states by using a cell membrane-based binding assay (Hoare et al., 2001)]. Unfortunately, none of the radioligands tested [[^I]-hTIP39,[^I]-I-mTIP39,[^I]-I-rPTH(1–34)] had a useable signal-to-background ratio for the rat PTH2 receptor in this assay.

HPTH(1–34) inclusion of the N-domain of the human PTH2 receptor in the rat receptor (hN-rPTH2) increased affinity (38 nM), equivalent to that of the human wild-type receptor (18 nM; Fig. 6A; Table 4). The affinity of hPTH(1–34) for the reciprocal chimera (rN-hPTH2, 220 nM) was not significantly different from that of the rat receptor (450 nM; Fig. 6A; Table 4). Experiments with the remaining chimeric receptors indicated that the inclusion of either EL1 or the C-terminal portion of the J-domain did not significantly affect hPTH(1–34) affinity, relative to the wild-type rat receptor into which these regions were substituted (Fig. 6, B and C; Table 4). Therefore, the N-domain, and not the J-domain, is the determinant of hPTH(1–34)’s human/rat PTH2 receptor binding selectivity in this assay.

The binding profile of hTIP39 for these chimeric receptors

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Fig. 5. hPTH(1–34) and hTIP39-stimulated cAMP accumulation via chimeric human/rat PTH2 receptors expressed in COS-7 cells. cAMP was measured as described under Materials and Methods. Data were normalized as a percentage of the maximal response to hTIP39, which was included as a reference at 1 μM concentration for each hPTH(1–34) concentration dependence assay. Data points are the mean ± range of duplicate measurements. The curves are fits to a four-parameter logistic equation. Data are from representative experiments that were performed three to seven times with similar results.
was more complex. Unexpectedly, combination of the human N-domain with the rat J-domain (hN-rPTH2) resulted in a significant increase of hTIP39 affinity (7.7 nM) compared with the wild-type human PTH2 receptor (26 nM; Fig. 6D; Table 4). The region of the rat PTH2 receptor J-domain involved in this effect was investigated further. Inclusion of the C-terminal portion of the J-domain (including EL2 and EL3) in combination with the human N-domain (hN→TM3-rPTH2) again resulted in a significantly higher affinity (7.0 nM) compared with the wild-type human PTH2 receptor (Fig. 6F; Table 4). However, the affinity-enhancing effect was not observed when EL1 of the rat receptor was incorporated into the human receptor (rEL1-hPTH2, IC<sub>50</sub> of 21 nM; Fig. 6E). These observations suggest that the C-terminal portion of the rat J-domain provides more hTIP39 binding energy than the equivalent region of the human PTH2 receptor, when in combination with the human N-domain. However, the reciprocal effect was not observed; inclusion of the entire C-terminal region of the human J-domain with the rat N-domain (rN-hPTH2 and rN→TM3-hPTH2) did not significantly affect hTIP39 binding affinity relative to the wild-type rat receptor (Fig. 6, D and F; Table 4). Differential TIP39 binding to the C-terminal portion of the J-domain therefore depends upon the host receptor. The combination of this region of the rat receptor with the N-domain of the human receptor increases affinity, whereas the combination of this region of the human receptor with the N-domain of the rat receptor has no effect. This suggests that the N-domain and C-terminal region of the J-domain act in concert to determine the hTIP39 binding affinity.

**Regions of Human and Rat PTH2 Receptors Specifying Differential bPTH(7–34) and hTIP(7–39) Binding.**

The rat PTH2 receptor binds available antagonist ligands with considerably lower affinity than the human receptor (Fig. 4; Table 2). We investigated the molecular basis of this affinity difference for [D-Tryp<sup>12</sup>]bPTH(7–34) and hTIP(7–39) by using the chimeric human/rat PTH2 receptors described above.

Two receptor regions partially specified the human/rat PTH2 receptor selectivity of [D-Tryp<sup>12</sup>]bPTH(7–34). Inclusion of the human N-domain or the human EL1 region in the rat receptor (hN-rPTH2 and hEL1-rPTH2) increased the affinity
Relative to rat PTH2 receptor (540 nM), but did not fully restore the binding affinity relative to the human receptor (53 nM) (Fig. 6, G and H; Table 4). Reciprocally, incorporation of the rat N-domain or EL1 in the human receptor (rN-hPTH2 and rEL1-hPTH2) reduced [D-Tyr12]-bPTH(7–34)’s affinity (210 and 300 nM, respectively) relative to the human receptor but did not fully reduce the affinity to that of the rat receptor (Fig. 6, G and H; Table 4). The combination of the N-domain and EL1 fully specified the human/rat PTH2 receptor binding affinity of [D-Tyr12]-bPTH(7–34). Inclusion of the human N-domain and EL1 in the rat receptor (hN–TM3-rPTH2) fully restored the binding affinity (54 nM), whereas the affinity for the reciprocal chimera (730 nM) was not significantly different from that of the rat receptor (Fig. 6I; Table 4). These findings indicate an approximately equal and additive contribution of EL1 and the N-domain to the differential [D-Tyr12]-bPTH(7–34) binding by human and rat PTH2 receptors.

**TABLE 4**

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<tr>
<th>Agonist and antagonist ligand binding affinity for wild-type and chimeric human/rat PTH2 receptors</th>
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<td>Displacement of 125I-TIP39 binding to COS-7 cells expressing the receptors was measured as described under Materials and Methods and the data was analyzed using a four-parameter logistic equation. Data are the mean ± S.E.M. from three to five experiments.</td>
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<tr>
<th>Receptor</th>
<th>hPTH(1–34)</th>
<th>hTIP39</th>
<th>hPTH(7–34)</th>
<th>hTIP(7–39)</th>
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<tbody>
<tr>
<td>hPTH2</td>
<td>7.74 ± 0.17&lt;sup&gt;a&lt;/sup&gt; (18)</td>
<td>7.58 ± 0.06&lt;sup&gt;c&lt;/sup&gt; (26)</td>
<td>7.28 ± 0.05&lt;sup&gt;e&lt;/sup&gt; (53)</td>
<td>7.18 ± 0.11&lt;sup&gt;f&lt;/sup&gt; (67)</td>
</tr>
<tr>
<td>rPTH2</td>
<td>6.34 ± 0.14&lt;sup&gt;b&lt;/sup&gt; (450)</td>
<td>7.04 ± 0.03&lt;sup&gt;d&lt;/sup&gt; (91)</td>
<td>6.27 ± 0.02&lt;sup&gt;f&lt;/sup&gt; (540)</td>
<td>6.09 ± 0.05&lt;sup&gt;b&lt;/sup&gt; (830)</td>
</tr>
<tr>
<td>hN-rPTH2</td>
<td>7.42 ± 0.08&lt;sup&gt;e&lt;/sup&gt; (38)</td>
<td>8.12 ± 0.07&lt;sup&gt;d&lt;/sup&gt; (7.7)</td>
<td>6.73 ± 0.07&lt;sup&gt;f&lt;/sup&gt; (190)</td>
<td>7.26 ± 0.03&lt;sup&gt;e&lt;/sup&gt; (55)</td>
</tr>
<tr>
<td>rN-hPTH2</td>
<td>6.66 ± 0.11&lt;sup&gt;c&lt;/sup&gt; (220)</td>
<td>6.98 ± 0.08&lt;sup&gt;d&lt;/sup&gt; (100)</td>
<td>6.69 ± 0.04&lt;sup&gt;f&lt;/sup&gt; (210)</td>
<td>6.52 ± 0.04&lt;sup&gt;c&lt;/sup&gt; (300)</td>
</tr>
<tr>
<td>hTM1–TM3-rPTH2</td>
<td>6.62 ± 0.12&lt;sup&gt;c&lt;/sup&gt; (240)</td>
<td>7.40 ± 0.07&lt;sup&gt;e&lt;/sup&gt; (40)</td>
<td>6.63 ± 0.04&lt;sup&gt;f&lt;/sup&gt; (230)</td>
<td>6.44 ± 0.05&lt;sup&gt;c&lt;/sup&gt; (370)</td>
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<tr>
<td>rTM1–TM3-hPTH2</td>
<td>7.28 ± 0.23&lt;sup&gt;a&lt;/sup&gt; (52)</td>
<td>7.68 ± 0.20&lt;sup&gt;e&lt;/sup&gt; (21)</td>
<td>6.52 ± 0.04&lt;sup&gt;f&lt;/sup&gt; (300)</td>
<td>6.92 ± 0.04&lt;sup&gt;c&lt;/sup&gt; (120)</td>
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<tr>
<td>hN–TM3-rPTH2</td>
<td>7.57 ± 0.06&lt;sup&gt;a&lt;/sup&gt; (27)</td>
<td>8.16 ± 0.10&lt;sup&gt;d&lt;/sup&gt; (7.0)</td>
<td>7.27 ± 0.09&lt;sup&gt;e&lt;/sup&gt; (54)</td>
<td>7.07 ± 0.05&lt;sup&gt;f&lt;/sup&gt; (85)</td>
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<tr>
<td>rN–TM3-hPTH2</td>
<td>6.66 ± 0.09&lt;sup&gt;c&lt;/sup&gt; (220)</td>
<td>6.95 ± 0.08&lt;sup&gt;d&lt;/sup&gt; (110)</td>
<td>6.14 ± 0.04&lt;sup&gt;f&lt;/sup&gt; (730)</td>
<td>6.04 ± 0.05&lt;sup&gt;e&lt;/sup&gt; (920)</td>
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For each ligand, statistical comparison of the data for the different receptors was performed using single-factor analysis of variance followed by post hoc analysis using the Newman-Keuls test. For all four ligands, significant difference was observed between the –logIC<sub>50</sub> values for the different receptors (p < 0.0001 for all ligands).

-<sup>a</sup>c<sup>e</sup> Significantly different from the IC<sub>50</sub> value of the corresponding ligand for the rat PTH2 receptor.

-<sup>b</sup>d<sup>f</sup> Significantly different from the IC<sub>50</sub> value of the corresponding ligand for the human PTH2 receptor.
[\text{-}[\text{Trypt}^{12}]\text{bPTH}(7\text{-}34)]$, but qualitatively different. Inclusion of the human N-domain within the rat receptor (hN-rPTH2) fully restored binding affinity (from 830 to 55 nM) relative to the wild-type human receptor (67 nM) (Fig. 6J; Table 4). The hTIP(7\text{-}39) binding affinity for the reciprocal chimera (rN-hPTH2) was significantly less (300 nM) than that of the wild-type rat receptor (830 nM), suggesting that the human J-domain partially contributes to the binding selectivity of this ligand (Fig. 6J; Table 4). EL1 was identified as the region responsible: Incorporation of human EL1 within the rat receptor (hEL1-rPTH2) partially increased the hTIP(7\text{-}39) affinity (from 830 to 370 nM), and the rat EL1 slightly but significantly decreased the affinity (from 67 to 120 nM) when included in the human PTH2 receptor (rEL1-hPTH2) (Fig. 6K; Table 4). The affinity of hTIP(7\text{-}39) for a receptor containing the human N-domain and EL1 (hN\text{--}TM3-rPTH2, 85 nM) was equivalent to that of the human receptor (Fig. 6L; Table 4). Inclusion of both the rat N-domain and EL1 (rN\text{--}TM3-hPTH2) reduced the binding affinity (920 nM) to that of the rat receptor (Fig. 6L; Table 4). These findings suggest that the N-domain and EL1 both contribute to the differential hTIP(7\text{-}39) binding of human and rat PTH2 receptors, but that the N-domain provides a larger contribution than EL1.

**Discussion**

Structure-activity and photochemical cross-linking studies have identified a common “two-site” orientation of ligand binding to PTH2 and PTH1 receptors (Bergwitz et al., 1996, 1997; Zhou et al., 1997; Bisello et al., 1998; Clark et al., 1998; Hannstaedt et al., 1998; Turner et al., 1998; Behar et al., 1999; Hoare et al., 2000a, 2001) and other type II G protein-coupled receptors (Holtmann et al., 1995; Stroop et al., 1995; Bergwitz et al., 1996; Couvineau et al., 1996; Dautzenberg et al., 1998). The extracellular N-terminal domain of the receptor (N-domain) binds the C-terminal portion of the ligand (N-interaction) and the J-domain binds the ligand’s amino-terminal portion (J-interaction). In this study we compared rat and human PTH2 receptor pharmacology by measuring ligand-stimulated increases of intracellular calcium and ligand binding. Included in this evaluation were mouse TIP39 (Hoare et al., 1999), consistent with a “closed” conformation postulated for the active state of the PTH1 receptor (Hoare et al., 2001). Interestingly, in this study the EL2/EL3 region was also a determinant of TIP39 interaction with the PTH2 receptor (Fig. 6, C and F), although this was evident as a contribution to binding affinity rather than signaling efficacy and was only evident for receptors containing the N-domain of the human PTH2 receptor.

The binding selectivity of PTH was measured using a whole-cell radioligand binding assay, which probably measures ligand affinity for inactive receptor states (Hoare and Usdin, 2001). The affinity of hPTH(1\text{-}34) for the inactive state of the PTH2 receptor was completely specified by the N-domain. The N-domain has been identified as a determinant of binding affinity for TIP39 binding to the PTH2 receptor (Hoare et al., 2000a). PTH selectivity for the active state of the receptor was evaluated using the EC_{50} for cAMP accumulation. Although this measurement incorporates ligand affinity for the active state of the receptor, it provides only an approximate readout because other processes can contribute to the EC_{50} (such as receptor reserve). None of the human PTH2 receptor regions restored high PTH potency when incorporated into the rat PTH2 receptor. Notably incorporation of the human N-domain (the affinity determinant) with EL1 or with EL2/EL3 (the efficacy determinants) failed to restore high PTH potency, i.e., the effect of these regions was not additive. Reciprocally, insertion of the rat N-domain, EL1, or EL2/EL3 into the human receptor de-
increased PTH(1–34) potency to that of the rat receptor. These findings indicate that multiple regions throughout the receptor are required in concert for the PTH-PTH2 receptor complex to form the high-affinity active state. This suggests that the global conformation of the rat PTH2 receptor prevents it from adopting the high-affinity active state when in complex with PTH.

Phylogenetic analysis of the PTH receptor family is consistent with a common ancestor of human and rat PTH2 receptors (Rubin and Jüppner, 1999). This suggests that the functional difference of PTH responsiveness resulted either from the human receptor maintaining a strong PTH response through evolution with a loss of this response in the rat, or that the human receptor has gained the ability to be activated by the ligand. The latter hypothesis is supported by the weak PTH efficacy at the zebrafish PTH2 receptor (Rubin and Jüppner, 1999; Hoare et al., 2000b). In this study we determined which parts of the PTH2 receptor are responsible for the differential PTH responsiveness of the human and rat receptors. As described above, at least three molecular determinants were identified, and these regions probably act in concert to produce different conformations of the two receptors when in complex with PTH. Based on this finding it is tempting to speculate that PTH activation of the human PTH2 receptor serves a physiological function.

Previously described antagonists that bind the human PTH2 receptor with moderate affinity [hTIP(7–39), [α-Trp]12]bPTH(7–34), and PTHrP(1–21)PTH(22–34)] bound the rat receptor with 10- to 30-fold lower affinity. [hTIP(7–39), shown to be an antagonist in camp assays, also did not stimulate increases of Ca2+ via the human PTH2 receptor (data not shown).] A new ligand, mTIP(7–39), was the highest affinity antagonist identified for the human PTH2 receptor (IC50 = 6.2 nM).125I-mTIP(7–39) provides the first radiolabeled antagonist for this receptor and should be useful for structure-function studies. mTIP(7–39) bound the rat receptor with low affinity (300 nM). A high-affinity antagonist for the rat PTH2 receptor would be useful for investigating the PTH2 receptor’s physiological role in rodents, so we investigated the molecular basis of weak antagonist affinity for the PTH2 receptor.

For both [α-Trp]12]bPTH(7–34) and hTIP(7–39) human/rat PTH2 receptor selectivity was determined by the N-domain and EL1. The N-domain of the receptor probably contributes direct interactions with these antagonist ligands: position 13 of PTH cross-links to residues 138 to 147 of the human receptor, at the C-terminal end of the N-domain (Behar et al., 1999). The N-domain of the PTH1 receptor binds bPTH(7–34) (Jüppner et al., 1994). It is not presently clear whether EL1 is directly involved in binding antagonist ligands, or indirectly, for example, by affecting the receptor conformation. A recent photochemical cross-linking study identified an interaction between parabenzyloxymethyl-conjugated Lys27 of PTH and Leu261 of the PTH1 receptor, suggesting a direct role of EL1 in receptor-ligand interaction (Greenberg et al., 2000). The EL2/3 region was not involved in specifying hPTH(7–34) and hTIP(7–39) binding, in striking contrast to this region’s involvement in binding full-length hPTH(1–34) and hTIP39. These considerations suggest that the loss of activation produced by amino-terminal truncation of the ligands involves an altered interaction with the J-domain, possibly a loss of interaction with the EL2/3 region of the receptor.

N-terminal truncation of hTIP39 and mTIP39 was attempted to develop a high-affinity antagonist. However, this truncation of TIP39 decreased the binding affinity for the rat PTH2 receptor (9-fold for hTIP39 and 40-fold for mTIP39) to a greater extent than for the human receptor (2.6- and 4.1-fold decrease, respectively). This suggests that interaction of the 1 to 6 portion of TIP39 with the J-domain contributes more binding energy for the rat receptor than for the human receptor. This hypothesis is supported by the increase of hTIP39 binding affinity produced by inserting the rat EL2/3 region into the human PTH2 receptor (Fig. 6 F). These considerations suggest two possibilities for the development of a high-affinity rat PTH2 receptor antagonist. 1) Increase the affinity of the N-interaction by modifying residues in the C-terminal portion of TIP(7–39). However, the utility of TIP(7–39) analogs might be limited due to their high-affinity antagonism of the PTH1 receptor (Hoare and Usdin, 2000; Jonsson et al., 2001). 2) Introduce modifications into the N-terminal region of TIP39 that eliminate activation without appreciably affecting binding affinity.

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