The parathyroid hormone (PTH)-1 receptor mediates the pathophysiological effects of PTH in hyperparathyroidism and PTH-related protein (PTHrP) in humoral hypercalcemia of malignancy. A PTH1 receptor antagonist may be of therapeutic utility in these disorders. We recently identified a novel antagonist, tuberoinfundibular peptide (7-39) [TIP(7-39)], derived from the likely endogenous ligand for the PTH2 receptor TIP39. In this study its in vitro profile is evaluated and compared with that of [Nle8,18,Tyr34]bPTH(7-34) and PTHrP(7-34), representing the two previously known structural classes of PTH1 receptor antagonists. TIP(7-39) binds with higher affinity (6.2 nM) to the PTH1 receptor than [Nle8,18,Tyr34]bPTH(7-34) (45 nM) and PTHrP(7-34) (65 nM) and displays a 5.5-fold greater PTH1/PTH2 receptor selectivity. TIP(7-39) does not stimulate cAMP accumulation via the PTH1 receptor [in a sensitive assay that detects the activity of the weak partial agonist [Nle8,18,Tyr34]bPTH(3-34)] and does not increase intracellular calcium. Schild analysis for TIP(7-39) was consistent with purely competitive antagonism of PTH(1-34)’s stimulation of cAMP accumulation (slope = 0.99 ± 0.24). The pKᵦ₅ for TIP(7-39) (7.1 ± 0.3) was higher than that for [D-Trp12,Tyr34]bPTH(7-34) (6.5 ± 0.0) and PTHrP(7-34) (6.0 ± 0.1). Binding of 125I-TIP(7-39) to the PTH1 receptor could be measured (Kᵦ₅ = 1.3 ± 0.1 nM, Bᵦ₅max = 1.3 ± 0.1 pmol/mg), whereas binding of 125I-[Nle8,18,D-Trp12,Tyr34]bPTH(7-34) could not be detected. Kinetic analysis indicated that 125I-TIP(7-39) dissociates much more slowly (t½ = 14 min) than [D-Trp12,Tyr34]bPTH(7-34) (13 s) and PTHrP(7-34) (9 s). The novel antagonist TIP(7-39) therefore displays a more favorable in vitro pharmacological profile than antagonists derived from PTH and PTHrP and may be useful for demonstrating the utility of PTH1 receptor antagonism in the treatment of hypercalcemia.

Received for publication May 19, 2000.

1 This study was supported by the National Institute of Mental Health, Intramural Research Program.

ABBREVIATIONS: PTH1, type 1 parathyroid hormone receptor; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; HMM, humoral hypercalcemia of malignancy; HPT, hyperparathyroidism; TIP39, tuberoinfundibular peptide of 39 residues; DMEM, Dulbecco’s modified Eagle’s medium; GTP-γ-S, guanosine-5′-O-(3-thio)triphosphate; DPBS, Dulbecco’s phosphate-buffered saline.
yielded PTH(7-34) and PTHrP(7-34) analogs with reduced signaling efficacy but at the expense of lower binding affinity (Rosenblatt et al., 1980; McGowan et al., 1983; McKee et al., 1988, 1990; Chorev et al., 1990; Goldman et al., 1998; Hoare et al., 1999a). The residue 7-34 fragments act as antagonists, or in some cases weak partial agonists, in vitro (Goldman et al., 1988; McKee et al., 1988, 1990; Chorev et al., 1990). These peptides can antagonize the effects of exogenous PTH or PTHrP in thyroparathyroidectomized rats (Horiiuchi et al., 1983; Doppelt et al., 1986; Horiiuchi and Rosenblatt, 1987; Dresner-Pollak et al., 1996). Administration of the antagonist before PTH or PTHrP exposure may be required to observe significant inhibition (Dresner-Pollak et al., 1996).

We identified a novel PTH1 receptor antagonist in our investigation of the receptor selectivity of tuberoinfundibular peptide of 39 residues (TIP39). This recently discovered hypothalamic peptide activates the PTH2 receptor and may be its natural ligand (Hoare et al., 1999b; Usdin et al., 1999). The human PTH2 receptor has 52% amino-acid sequence identity to the human PTH1 receptor (Usdin et al., 1995). TIP39 shares some sequence homology with PTH and PTHrP; five residues are identical when the sequences of TIP39, PTH, and PTHrP are aligned (Usdin, 2000). TIP39 strongly activates the PTH2 receptor and binds to it with subnanomolar affinity (0.59 nM) (Hoare et al., 2000). TIP39 binds to the PTH1 receptor with moderate affinity (59 nM) but produces little or no stimulation of cAMP accumulation. Deletion of six residues from the N terminal of TIP39 reduces binding affinity for the PTH2 receptor by 72-fold but increases PTH1 receptor affinity by a factor of 10 (Hoare et al., 2000). TIP(7-39) does not detectably stimulate cAMP accumulation at PTH1 or PTH2 receptors. Therefore, TIP(7-39) is a selective, high-affinity antagonist for the PTH1 receptor.

TIP(7-39) may possess different properties from N-terminally truncated PTH and PTHrP analogs, owing to its different primary structure and because the parent peptide, unlike PTH and PTHrP, does not appreciably activate the receptor. We have now evaluated the in vitro functional and binding properties of bovine TIP(7-39) at the PTH1 receptor and compared its pharmacological profile with that of [α-Trp12,Tyr24]bovine PTH(7-34) and PTHrP(7-34), which represent the two previously known structural classes of PTH1 receptor antagonists.

**Materials and Methods**

**Reagents and Peptides.** The following peptides were obtained from Bachem (Torrance, CA) or Peninsula Laboratories (Belmont, CA): [α-Trp12,Tyr24]bPTH(7-34) amide, [Nle8,18-α-Trp12,Tyr24]bPTH(7-34) amide, PTHrP(7-34) amide, rPTH(1-34), [Nle8,21,Tyr34]rPTH(1-34) amide, and [Nle8,18,Tyr34]bPTH(3-34) amide. The letters “b” and “r” designate the peptide sequence as bovine and rat, respectively. These peptides were dissolved in 10 mM acetic acid at a concentration of 1 mM, calculated using the peptide content and weight provided by the supplier. βTIP39 and βTIP(7-39) were purchased from Biomolecules Midwest (Waterloo, IL). βTIP(7-39) was quantified using the copper bicinchoninic acid method (Pierce, Rockford, IL) with TIP39 as the standard before PTH or PTHrP exposure may be required to observe significant inhibition (Dresner-Pollak et al., 1996).

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was measured in the presence of 10 μM GTPγS (to measure antagonist affinity for the G-protein-uncoupled state of the receptors). A, disappearance of 125I-[Nle8,18,Tyr34]PTH(1-34) binding to the PTH1 receptor. B, disappearance of 125I-TIP39 binding to the PTH2 receptor. ○, TIP(7-39); □, [n-Trp12,Tyr34]PTH(7-34); △, PTHrP(7-34). Nonspecific binding was measured in the presence of 300 nM unlabeled analog of the radioligand. For these representative experiments total binding of 125I-[Nle8,18,Tyr34]PTH(1-34) varied from 4,400 to 4,800 cpm, nonspecific binding ranged from 1,900 to 2,400 cpm, and the total radioligand added was 43,000 cpm. The ranges of total and nonspecific binding for 125I-TIP39 were 3,600 to 3,900 cpm and 570 to 1,000 cpm, respectively, and the total radioligand added was 46,000 cpm. Data points are the mean ± S.E. of triplicate measurements. The data are from representative experiments that were performed three times except for measurement of [n-Trp12,Tyr34]PTH(7-34) binding to the PTH2 receptor, which was performed twice.

were brought to 21°C by incubation in a water bath for 15 min. Prewarmed membranes were then added to the wells at various time points and the assay wells harvested simultaneously. Nonspecific binding in these experiments was defined using 300 nM unlabeled TIP(7-39), incubated with membranes and radioligand for 1 and 60 min. In the experiment in Fig. 7, a second, unlabeled ligand was included in the assay incubation to estimate the association and dissociation rate constants of the unlabeled ligand (see below). In dissociation experiments radioligand and membranes were equilibrated for 60 min before addition of unlabeled TIP(7-39) (300 nM final concentration) at various time points. All time points were harvested simultaneously. (As a result the shorter time points of the time course were equilibrated with radioligand for between 1 and 2 h.) Nonspecific binding was defined using 300 nM unlabeled TIP(7-39), which was included in the equilibration phase of the assay.

Measurement of Intracellular Calcium Concentration. HEK293 cells stably expressing the PTH1 receptor were seeded in a 96-well plate at 100,000 cells/well. The following day, medium was removed and the cells washed once with 0.1 ml of Dulbecco's phosphate-buffered saline (DPBS) containing 1 mM Ca2+ and 1 mM Mg2+. Cells were then loaded with 5 μM fluo-4 acetoxymethyl ester, with 0.1% (w/v) Pluronic F-127 and 2.5 mM probenecid in DPBS for 1 h at 37°C. After two washes with DPBS supplemented with 0.1% BSA, cells were incubated in 0.1 ml of the same buffer for 30 min at 37°C. This buffer was then removed and 50 μl of prewarmed DPBS with BSA added. Baseline fluorescence was then measured for 80 s at 37°C in a Cytofluor 4000 multwell plate fluorimeter (PerSpective Biosystems, Framingham, MA) (excitation wavelength 485 ± 20 nm, emission wavelength 530 ± 25 nm). Test agents were then added and fluorescence monitored as before. Fluorescence was measured in duplicate wells of cells for each experimental condition. Cytosolic free calcium concentration ([Ca2+]i) was calculated using the following equation: [Ca2+]i = Kd(F - Fmin)/(Fmax - F) where Kd is the ion dissociation constant (345 nM) for the indicator and F the fluorescence signal in arbitrary units. Fmax (maximum fluorescence at Ca2+ saturation of the indicator) was determined by addition of 130 μM ionomycin and Fmin (background fluorescence) measured after addition of 20 μM EGTA.

Fig. 2. Effect of TIP(7-39) on cAMP accumulation in COS-7 cells expressing a C-terminal-modified human PTH1 receptor. The PTH1 receptor was modified by addition of a 12-amino-acid residue hemagglutinin epitope to the C terminus. cAMP accumulation was measured in response to rPTH(1-34) () [Nle8,18,Tyr34]PTH(3-34) (○), and TIP(7-39) (□) as described under Materials and Methods. The basal accumulation of cAMP was 0.95 ± 0.04 pmol/well and the accumulation in the presence of a 320 nM rPTH(1-34) was 4.5 ± 0.6 pmol/well (n = 3). Data points are the mean ± range of duplicate measurements. (Where error bars are not apparent they are smaller than the symbols.) The experiment for [Nle8,18,Tyr34]PTH(3-34) was performed five times with similar results. The assay for TIP(7-39) was performed three times and in each experiment linear regression analysis indicated that the gradient was not significantly different from zero (P values of .54, .16, and .09).

Measurement of Intracellular Calcium Concentration. HEK293 cells stably expressing the PTH1 receptor were seeded in wells of a 96-well plate at 100,000 cells/well. The following day, medium was removed and the cells washed once with 0.1 ml of Dulbecco’s phosphate-buffered saline (DPBS) containing 1 mM Ca2+ and 1 mM Mg2+. Cells were then loaded with 5 μM fluo-4 acetoxymethyl ester, with 0.1% (w/v) Pluronic F-127 and 2.5 mM probenecid in DPBS for 1 h at 37°C. After two washes with DPBS supplemented with 0.1% BSA, cells were incubated in 0.1 ml of the same buffer for 30 min at 37°C. This buffer was then removed and 50 μl of prewarmed DPBS with BSA added. Baseline fluorescence was then measured for 80 s at 37°C in a Cytofluor 4000 multwell plate fluorimeter (PerSpective Biosystems, Framingham, MA) (excitation wavelength 485 ± 20 nm, emission wavelength 530 ± 25 nm). Test agents were then added and fluorescence monitored as before. Fluorescence was measured in duplicate wells of cells for each experimental condition. Cytosolic free calcium concentration ([Ca2+]i) was calculated using the following equation: [Ca2+]i = Kd(F - Fmin)/(Fmax - F) where Kd is the ion dissociation constant (345 nM) for the indicator and F the fluorescence signal in arbitrary units. Fmax (maximum fluorescence at Ca2+ saturation of the indicator) was determined by addition of 130 μM ionomycin and Fmin (background fluorescence) measured after addition of 20 μM EGTA.
Data Analysis. Concentration-dependence data for ligand-stimulated cAMP accumulation and inhibition of radioligand binding (Figs. 1, 2, 4, and 5) were analyzed using the following four-parameter logistic equation using Prism 2.01 (GraphPad Software Inc., San Diego, CA):

\[ y = \min + (\max - \min)/(1 + 10^{\log\min - \log\max}) \]  

where \( X \) is the logarithm of the ligand concentration and \( n \) is Hill slope. For cAMP accumulation data, \( y \) is the amount of cAMP produced 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 EC_{50}. For inhibition of radioligand binding, \( y \) is the cpm bound at a given unlabeled ligand concentration, \( \min \) is nonspecific binding and \( \max \) is total binding (the level of binding in the absence of unlabeled ligand), and \( K \) is the IC_{50}.

The effect of TIP(7-39) on rPTH(1-34)-stimulated cAMP accumulation at the human PTH1 receptor was analyzed using Schid analysis (Fig. 4), using the following equation:

\[ \log(DR - 1) = n \cdot \log[\text{antagonist}] + pA_2 \]  

where DR is the dose ratio (EC_{50} in the presence of antagonist divided by EC_{50} in the absence of antagonist), \( n \) is the gradient, and \( pA_2 \) is a measure of the antagonist potency. The \( pA_2 \) was subsequently converted to a \( pK_B \) value by fixing \( n \) at unity in the linear regression analysis.

\[ 125\text{I}-\text{TIP}(7-39) \]  

saturation of the PTH1 receptor was analyzed as follows. First, nonspecific binding [measured in the presence of 1 \( \mu \text{M} \) TIP(7-39)] was estimated as a fraction of the free radioligand concentration by linear regression. The values of \( K_D \) and \( B_{\max} \) were obtained by fitting total binding data (measured in the absence of unlabeled ligand) to the following equation using Prism 2.01:

\[ \text{Total binding} = c \cdot [L] + \frac{B_{\max} \cdot [L]}{K_D + [L]} \]  

where \( c \) is nonspecific binding expressed as a fraction of the free radioligand concentration. \( c \) was fixed at the previously determined value from the analysis of nonspecific binding values. The free radioligand concentration was calculated by subtracting either the nonspecific binding value or the total binding value from the total radioligand concentration.

\[ 125\text{I}-\text{TIP}(7-39) \]  

association data (total binding) were fitted to a biexponential association equation to account for association to specific and nonspecific sites (Fig. 7). This procedure was used because the value of nonspecific binding measured after 60 min was slightly greater than the value measured after 1 min. In the analysis the equilibrium level of nonspecific binding was fixed at that measured at 60 min. The observed association rate constant for nonspecific binding was high (>2 \( \text{min}^{-1} \)). The observed association rate of specific radioligand (\( L \)) binding \( k_{\text{on(specific)}} \) was fitted by linear regression to the equation \( k_{\text{on(specific)}} = k_{\text{on}} + k_{\text{m}}[L] \) where \( k_{\text{on}} \) and \( k_{\text{m}} \) are the association and dissociation rate constants, respectively. \[ 125\text{I}-\text{TIP}(7-39) \]  

dissociation data were fitted to a monoeponential dissociation equation. A biexponential equation did not significantly improve the fit in all cases (\( P > .7 \)).

The association and dissociation rate constants of unlabeled ligands were determined using the method devised by Motulsky and Mahan (1984) in which association of a radiolabeled antagonist \[ 125\text{I}-\text{TIP}(7-39) \]  

is measured in the presence of a fixed concentration of the unlabeled ligand. The model assumes that the ligands bind in a competitive manner according to simple bimolecular reactions. The total amount of radioligand bound to the receptor \( (RL) \) as a function of time was fitted to the following equation using SigmaPlot 3.0 (Jandel Scientific, SPSS Inc., Chicago, IL):

\[
[RL] = \frac{B_{\text{max}} k_{\text{L}}[L]}{K_D - K_S} \left[ \frac{k_{\text{on}}(K_D - K_S)}{K_D K_S} \frac{(k_4 - K_S)}{K_P} \exp(-K_S t) \right. \\
- \left. \frac{(k_4 - K_S)}{K_S} \exp(-K_S t) \right] + bg(1 - \exp(-k_4t))
\]

where

\[ K_A = k_1[L] + k_2 \]

\[ K_B = k_3[I] + k_4 \]

\[ K_P = 0.5[(K_A + K_B + \sqrt{(K_A - K_B)^2 + 4k_3k_4[L][I]})] \]

\[ K_S = 0.5[(K_A + K_B - \sqrt{(K_A - K_B)^2 + 4k_3k_4[L][I]})] \]

\( k_1 \) and \( k_4 \) are the association rate constants of the radioligand (\( L \)) and unlabeled ligand (\( I \)), respectively; \( k_3 \) and \( k_4 \) are the dissociation rate constants of the radioligand and unlabeled ligand, respectively; \( B_{\text{max}} \) is the total concentration of receptors; \( bg \) is nonspecific radioligand binding in cpm; and \( k_{\text{m}} \) is the observed association rate constant for nonspecific binding of radioligand. All parameters except \( k_3 \), \( k_4 \), and \( k_{\text{m}} \) were held constant in the analysis. \( B_{\text{max}} \) was calculated using the equilibrium level of specific \[ 125\text{I}-\text{TIP}(7-39) \]  

binding (measured in parallel in each experiment), the concentration of radioligand, and the kinetically derived radiodigand \( K_D \), using the specific binding component of eq. 3.

Statistical comparison of multiple means was performed initially by single-factor analysis of variance followed by post hoc analysis with the Newman-Keuls test. Statistical comparison of two means was performed using a two-tailed Student’s \( t \) test.

Results

Binding of Antagonists to the Human PTH1 and PTH2 Receptors. Radioligand binding assays were used to compare the receptor binding affinity of TIP(7-39) with that of \([\text{D-Trp}^{12},\text{Tyr}^{34}]\text{PTH}(7-34)\) and \([\text{PTHrP}^{34}]\text{PTH}(7-34)\). Membranes prepared from HEK293 cells expressing the human PTH1 receptor were labeled with \[ 125\text{I}-[\text{Nle}^{8,18},\text{Tyr}^{34}]\text{PTH}(1-34)\] and from HEK293 cells expressing the human PTH2 receptor with \[ 125\text{I}-\text{TIP}(39)\]. Binding was measured in the presence of 10 \( \mu \text{M} \) GTPγS to minimize complications arising from receptor-G-protein coupling, such as pseudoreversible binding of the agonist radiodigand (Hoare et al., 1999a).

Binding of all ligands to both receptors was described by a pseudo Hill slope of approximately unity (Table 1), consistent with a simple bimolecular reaction for the receptor-ligand interaction. TIP(7-39) bound with a significantly higher affinity to the PTH1 receptor than \([\text{D-Trp}^{12},\text{Tyr}^{34}]\text{PTH}(7-34)\) or \([\text{PTHrP}^{34}]\text{PTH}(7-34)\) (Fig. 1A; Table 1). The difference of IC_{50} was 7.3-fold for \([\text{D-Trp}^{12},\text{Tyr}^{34}]\text{PTH}(7-34)\) and 10-fold for \([\text{PTHrP}^{34}]\text{PTH}(7-34)\). All of the antagonist ligands bound with lower affinity to the PTH2 receptor than the PTH1 receptor (Fig. 1B; Table 1). However, TIP(7-39) displayed a 5.5-fold greater selectivity for the PTH1 receptor than \([\text{D-Trp}^{12},\text{Tyr}^{34}]\text{PTH}(7-34)\) or \([\text{PTHrP}^{34}]\text{PTH}(7-34)\) (Table 1).

Effect of TIP(7-39) on cAMP Accumulation in COS-7 Cells Expressing a C-Terminal Hemagglutinin-Tagged Human PTH1 Receptor. Some PTH1 receptor ligands that were initially identified as antagonists based on inhibition of PTH-stimulated cAMP accumulation have since been demonstrated to possess significant efficacy in more sensitive assay systems. The best characterized example is \([\text{Nle}^{8,18},\text{Tyr}^{34}]\text{bPTH}(3-34)\). TIP(7-39) did not detectably stim-
TABLE 1  
Comparison of antagonist binding to human PTH1 and PTH2 receptors 

<table>
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<tr>
<th>Ligand</th>
<th>PTH1 Receptor</th>
<th>PTH2 Receptor</th>
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<td></td>
<td>−logIC_{50}</td>
<td>Pseudo Hill</td>
</tr>
<tr>
<td></td>
<td>(IC_{50}, nM)</td>
<td>Slope</td>
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<tr>
<td>TIP39(7-39)</td>
<td>8.21 ± 0.07</td>
<td>1.30 ± 0.16</td>
</tr>
<tr>
<td>[Nle^{6,18},Tyr^{24}]PTH(3-34)</td>
<td>7.35 ± 0.22</td>
<td>1.10 ± 0.03</td>
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<tr>
<td>PTHrP(7-34)</td>
<td>7.19 ± 0.08</td>
<td>0.93 ± 0.24</td>
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Statistical significance between the mean IC_{50} value for the three peptides at the PTH1 receptor was tested by single-factor analysis of variance followed by post hoc analysis with the Newman-Keuls test: *P < .01; **P < .005.

The antagonist did not significantly affect the accumulation of cAMP in HEK293 expressing the human PTH1 receptor (Hoare et al., 2000) but in these cells a response to [Nle^{6,18},Tyr^{24}]bPTH(3-34) was also not detected (Hoare et al., 1999a). We attempted to develop a more sensitive measure of PTH1 receptor activation to evaluate the potential agonism of TIP(7-39), and used the ability to detect the partial agonism of [Nle^{6,18},Tyr^{24}]bPTH(3-34) as the criteria for this assay. In COS-7 cells expressing the wild-type PTH1 receptor a measurable cAMP response to [Nle^{6,18},Tyr^{24}]bPTH(3-34) was observed in two of five assays (data not shown). However, a hemagglutinin-tagged PTH1 receptor was detectably activated by this ligand in COS-7 cells in each of five experiments, with an E_{max} of 26 ± 4% of the maximal response to rPTH(1-34) (Fig. 2). This receptor contains a 12-amino-acid residue hemagglutinin epitope inserted at the C terminus (Clark et al., 1998.) TIP(7-39) did not detectably stimulate cAMP accumulation in this assay (Fig. 2): Linear regression analysis indicated that the slope defining the concentration dependence of cAMP accumulation was not significantly different from zero in three independent experiments. In addition, the level of cAMP accumulation produced by 3.2 μM TIP(7-39) (0.91 ± 0.04 pmol/well) was not significantly different (P = .51) from the accumulation measured in the absence of ligand (0.95 ± 0.04 pmol/well).

Effect of TIP(7-39) on Intracellular Calcium Concentration. The PTH1 receptor has been demonstrated to couple to other second messenger pathways in addition to stimulation of cAMP accumulation (Abou-Samra et al., 1992; Azarani et al., 1996; Friedman et al., 1999). One of the best studied of these additional pathways is the elevation of [Ca^{2+}]. We therefore tested whether TIP(7-39) affects [Ca^{2+}], using fluo-4-loaded HEK293 cells expressing the human PTH1 receptor. No change in [Ca^{2+}] was observed when these cells were incubated with a high concentration of TIP(7-39) (1 μM), whereas 3 nM rPTH(1-34) produced a robust, rapid, and transient increase in [Ca^{2+}] (Fig. 3). TIP(7-39) (1 μM) antagonized the effect of rPTH(1-34) (3 nM); the peak [Ca^{2+}] increase was reduced by 79 ± 1% and the rate of increase was reduced (Fig. 3).

Measurement of Antagonist Potency of TIP(7-39) at Human and Rat PTH1 Receptors Expressed in COS-7 Cells. Schild analysis of TIP(7-39) inhibition of rPTH(1-34)-stimulated cAMP accumulation was performed to examine the mechanism of action of the antagonist at the PTH1 receptor and to measure antagonist potency in a functional assay. TIP(7-39) produced a parallel rightward shift of the rPTH(1-34) concentration dependence curve for stimulation of cAMP production at the human PTH1 receptor (Fig. 4A). The antagonist did not significantly affect the E_{max} for rPTH(1-34) and did not detectably activate cAMP accumulation in the absence of agonist (see legend to Fig. 4). The Schild slope was 0.99 ± 0.24 (Fig. 4B). These observations strongly suggest that TIP(7-39) acts as a competitive antagonist of rPTH(1-34)-stimulated cAMP accumulation at the human PTH1 receptor, at least over the range of antagonist concentrations tested. The pK_{B} of TIP(7-39) at the human PTH1 receptor was 6.83 (150 nM). This value is 24-fold greater than the IC_{50} of TIP(7-39) for inhibition of [^{125}I]-rPTH(1-34) binding to the human PTH1 receptor (Table 1). TIP(7-39) also antagonized PTHrP(1-34)-stimulated cAMP accumulation at the human PTH1 receptor, with a pK_{B} of 6.94 ± 0.09 (110 nM) (graphical data not shown). The pK_{B} of the antagonist was also measured for the rat PTH1 receptor expressed in COS-7 cells, using 3.2 μM TIP(7-39) and rPTH(1-34) as the agonist (graphical data not shown). The pK_{B} value of 6.51 ± 0.23 (310 nM) was not greatly different from that for the human PTH1 receptor.

Antagonist Potency in the Presence of Human Plasma. One explanation that has been proposed for the lack of effect of PTH1 receptor antagonists in vivo is inactivation...
of the ligand as a result of ligand binding to plasma proteins (Kukreja et al., 1994). We investigated this possibility by measuring the shift of rPTH(1-34) EC$_{50}$ produced by the antagonist in the absence and presence of 20% human plasma. It is important to note that this experiment does not address the effects of serum proteases on the antagonist effect because protease inhibitors were included in the assay. Human plasma did not reduce the antagonist potency of TIP(7-39) (Fig. 5), [D-Trp$_{12}$,Tyr$_{34}$]rPTH(7-34), or PTHrP(7-34) (Table 2). Indeed, plasma increased antagonist potency between 2.3- and 3.5-fold (Table 2). These experiments also demonstrate that TIP(7-39) displays a greater antagonist potency than either [D-Trp$_{12}$,Tyr$_{34}$]rPTH(7-34) or PTHrP(7-34), in both the absence and presence of plasma (Table 2).

**Binding of $^{125}$I-TIP(7-39) to the Human PTH1 Receptor in HEK293 Cell Membranes.** To enable a more detailed characterization of its ligand binding mechanism we prepared radiolabeled TIP(7-39). bTIP39 contains a tyrosine residue at position 29 and a methionine residue at position 30 (Usdin et al., 1999), so $^{125}$I-TIP39(7-39) was prepared using the mildly oxidizing lactose peroxidase method. Specific binding of this radioligand was detected in membranes prepared from HEK293 cells expressing the human PTH1 receptor (using 300 nM TIP(7-39) or 300 nM TIP39 to define nonspecific binding), whereas no specific binding was detected in HEK293 membranes prepared from nontransfected cells (data not shown). The total binding/nonspecific binding ratio for $^{125}$I-TIP(7-39) was approximately 5:1, which is comparable with the signal-to-noise ratio of 6:1 obtained with $^{125}$I-[Nle$_{8}$,18,Tyr$_{34}$]bPTH(3-34) (a commonly used radiolabeled antagonist/partial agonist for the PTH1 receptor). The affinity of $^{125}$I-TIP(7-39) for the PTH1 receptor was measured in saturation experiments, using varying concentrations of the radioligand. The saturation data were fitted well by a single-site saturation isotherm (Fig. 6A), a two-site model not providing a significant improvement to the fit (P values ranged from 0.75 to 0.95). The $K_D$ for $^{125}$I-TIP39(7-39) was 1.3 ± 0.1 nM and the $B_{max}$ was 1.3 ± 0.1 pmol/mg (n = 3). This $K_D$ is comparable with that for [Nle$_{8}$,18,Tyr$_{34}$]bPTH(3-34) (2.0 nM, Hoare and Usdin, 1999). The $B_{max}$ is slightly higher than that for [Nle$_{8}$,18,Tyr$_{34}$]bPTH(3-34) (0.7 pmol/mg, Hoare and Usdin, 1999). However this value was obtained from homologous displacement experiments, which may be less accurate than saturation experiments for measurement of $B_{max}$ if there is a difference between the binding affinities of the iodinated and noniodinated ligands.

**Measurement of Antagonist Binding Kinetics at the Human PTH1 Receptor in HEK293 Cell Membranes.** The association and dissociation rate constants for $^{125}$I-TIP(7-39) binding to the PTH1 receptor were measured directly using data from the time courses of radioligand association and dissociation. The affinities of [D-Trp$_{12}$,Tyr$_{34}$]rPTH(7-34) and PTHrP(7-34) are probably too low to permit their use as radio-
TABLE 2
Effect of human plasma on the potency of antagonist ligands for inhibition of PTH(1-34)-stimulated cAMP accumulation at the human PTH1 receptor.

The human PTH1 receptor was transiently expressed in COS-7 cells and cAMP determined as described under Materials and Methods. Plasma, antagonist, and varying concentrations of agonist were added to the cells in rapid succession and the cells incubated for 40 min at 37°C. The effect of the antagonist on the rPTH(1-34) EC50 and Emax was determined using 1 μM TIP(7-39), 1 μM [α-Trp12,Tyr34]PTH(7-34), and 3.2 μM PTHrP(7-34). The values are mean ± range from two experiments. Plasma produced little effect on cAMP accumulation in the absence of agonist (1.6 ± 0.02 and 1.92 ± 0.08 pmol/well in the absence and presence of plasma, respectively). The −logEC50 for rPTH(1-34) was 9.34 ± 0.04 and 9.63 ± 0.13 in the absence and presence of 20% plasma, respectively. The rPTH(1-34) Emax in the presence of 20% plasma was 88 ± 21% of the Emax in the absence of plasma.

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<tr>
<th>Antagonist</th>
<th>No Plasma</th>
<th>% Maximal rPTH Response</th>
<th>20% Plasma</th>
<th>% Maximal rPTH Response</th>
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<td>7.13 ± 0.27</td>
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Discussion

The PTH1 receptor is involved in disorders of calcium metabolism because it is the site of action of PTH and PTHrP. HMM resulting from bone resorption can be effectively treated in the long term using bisphosphonates, which inhibit resorptive processes (Singer et al., 1991; Brown and Robbins, 1999). However, the effect of these compounds is not evident until several days after treatment is initiated (Singer et al., 1991). An alternative strategy in development is neutralization of the osteoclast differentiation factor osteoprotegerin ligand by osteoprotegerin (Capparelli et al., 2000), but neither of these antiresorptive approaches target the renal effects of the PTH1 receptor. HPT can be treated surgically by parathyroidectomy but medical therapy may be required to stabilize blood calcium levels before surgery or for patients who cannot be treated surgically. Calcimimetic compounds have been proposed as potential therapies for primary HPT (Nemeth and Fox, 1999). Despite these advances, effective medical treatments for acute hypercalcaemic crisis and primary HPT are lacking. PTH1 receptor antagonism may provide an alternative or complementary therapeutic strategy. However, PTH1 receptor antagonists based on the structure of PTH or PTHrP have so far not been effective (Kukreja et al., 1994; Rosen et al., 1997).

In this study we investigated the functional properties of a novel PTH1 receptor antagonist, TIP(7-39) (Hoare et al., 2000). The effects were compared with those of previously described antagonists produced by N-terminal truncation of PTH (α-Trp12,Tyr34[PTH(7-34)] and PTHrP [PTHrP(7-34)]. The principal findings of this study are as follows: 1) TIP(7-39) acts as a purely competitive antagonist of the PTH1 receptor at the concentrations tested. 2) TIP(7-39) binds with higher affinity to the PTH1 receptor than α-Trp12,Tyr34[PTH(7-34)] or PTHrP(7-34) and displays a greater PTH1/PTH2 receptor selectivity. 3) Human plasma did not reduce the potency of any of the antagonists in the presence of protease inhibitors. 4) Specific binding of 125I-TIP(7-39) to the PTH1 receptor can be measured and is
well described by a simple bimolecular reaction. 5) The dissociation rate constant of $^{125}$I-TIP(7-39) is considerably lower than that of the previously described antagonist ligands. The higher PTH1 receptor binding affinity of TIP(7-39) indicates that the ligand may hold more promise for the development of highly potent, selective PTH1 receptor antagonists than PTH- or PTHrP-based peptides. The benefit of enhanced PTH1/PTH2 receptor-binding selectivity is not clear at present but such selectivity should minimize side effects resulting from blockade of the PTH2 receptor.

Fig. 6. Binding of $^{125}$I-TIP(7-39) to the human PTH1 receptor. Radiolabeled TIP(7-39) was prepared and measurement of radioligand binding to the PTH1 receptor in HEK293 cell membranes performed as described under Materials and Methods. A, $^{125}$I-TIP(7-39) saturation of the PTH1 receptor in HEK293 cell membranes performed as described under Materials and Methods. The time course of radioligand association with the PTH1 receptor in HEK293 cell membranes was measured as described under Materials and Methods, in the absence of unlabeled ligand (●) or in the presence of 60 nM [D-Trp$^{12}$,Tyr$^{34}$]PTH(7-34) (○), 100 nM PTHrP(7-34) (Δ), or 3 nM [Nle$^{8}$,Tyr$^{34}$]PTH(3-34) (□). Association time course data in the presence of unlabeled ligand were fitted to eq. 4 to obtain estimates of $k_{on}$ and $k_{off}$, respectively, the association and dissociation rate constants of the unlabeled ligand. In this experiment the following parameters were held constant in the analysis: $B_{max} = 31,500$ cpm, $[L] = 9.28 \times 10^{-11}$ M, $b_{g} = 596$ cpm, $k_{1} = 8.9 \times 10^{4} \text{M}^{-1} \text{min}^{-1}$, $k_{2} = 0.051 \text{min}^{-1}$, $[J]$ as given above. The curves are the best fits to the data. The slight overshoot observed for $^{125}$I-TIP(7-39) association in the presence of [Nle$^{8}$,Tyr$^{34}$]PTH(3-34) is fitted well by eq. 4, arising from a lower value of $k_{2}$ than $k_{1}$ (Motulsky and Mahan, 1984). The data points are the mean ± S.E. from triplicate determinations. Data are from a representative experiment. The experiments were performed twice (for [Nle$^{8}$,Tyr$^{34}$]PTH(3-34)) or three times (for the other two ligands) with very similar results. In most cases the error bars are enclosed within the symbol.

TABLE 3

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$k_{on}$</th>
<th>$k_{off}$</th>
<th>$k_{on}/k_{off}$</th>
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<td>$^{125}$I-TIP(39)</td>
<td>$8.9 \pm 3.0$</td>
<td>$0.051 \pm 0.001$</td>
<td>$14 \text{min}$</td>
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<tr>
<td>[D-Trp$^{12}$,Tyr$^{34}$]PTH(7-34)</td>
<td>$13 \pm 1$</td>
<td>$3.2 \pm 0.3$</td>
<td>$25 \pm 2$</td>
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<tr>
<td>PTHrP(7-34)</td>
<td>$8.5 \pm 2.2$</td>
<td>$4.5 \pm 0.4$</td>
<td>$55 \pm 16$</td>
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<tr>
<td>[Nle$^{8}$,Tyr$^{34}$]PTH(3-34)</td>
<td>$3.1 \pm 0.1$</td>
<td>$0.030 \pm 0.011$</td>
<td>$0.98 \pm 0.36$</td>
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TIP(7-39) acts as a competitive antagonist of the PTH1 receptor at the concentrations used in this study: in assays of cAMP accumulation the peptide produces a rightward-shift of the agonist concentration-dependence curve, defined by a Schild plot slope of unity, and it does not affect the maximal stimulation produced by the agonist (Fig. 4). In [Ca$^{2+}$], assays the ligand strongly inhibits the response to rPTH(1-34) (Fig. 3). TIP(7-39) also appears to be a pure antagonist at the PTH1 receptor within the detection limits of the assays used.
The ligand does not significantly activate the hemagglutinin-tagged human PTH1 receptor expressed in COS-7 cells, a highly sensitive assay in which the partial agonism of [Nle\(^{6,18}\),Tyr\(^{34}\)]bPTH(3-34) can be detected (Fig. 2). The greater sensitivity of this assay compared with that for the PTH1 receptor in HEK293 cells could be a result of a higher level of receptor expression in COS-7 cells (approximately 10\(^5\) and 10\(^6\) receptors/cell, respectively). The variable results obtained for the wild-type PTH1 receptor in COS-7 cells may be a result of variable transfection efficiency. The more consistent response observed with the C-terminally modified tagged receptor versus the wild-type receptor may be result from altered receptor-G-protein coupling (Iida-Klein et al., 1995).

We found that the functional potency of the antagonist ligands was markedly less than the affinity of the ligands measured in radioligand binding assays. This observation is common in studies of PTH1 receptor antagonism (Goldman et al., 1988; McKee et al., 1988). To an extent this effect may be due to the different assay conditions used. In the cAMP accumulation assay a 37°C preincubation of TIP(7-39) with receptor before addition of the agonist reduced the antagonist potency compared with simultaneous addition of the ligands (K\(_B\) values of 310 and 74 nM, respectively). This finding could be explained by degradation of the peptide in the longer incubation with the cells. The functional potency of TIP(7-39) was further increased by the addition of plasma (K\(_B\) of 21 nM), which could block nonspecific binding more effectively. In contrast the radioligand binding assay used to measure antagonist binding affinity was designed to minimize ligand degradation and nonspecific binding (Hoare and Usdin, 1999). The remaining discrepancy could be explained by a lack of equilibration in the adenylyl cyclase assay, owing to the slow dissociation of rPTH(1-34) from the PTH1 receptor (Hoare et al., 1999a). Alternatively, the discrepancy may be a result of the different environments of the receptor in the two assays (cell membranes versus whole cells).

Inactivation by binding to plasma proteins has been proposed to explain the lack of in vivo efficacy of PTH1 receptor antagonists. In a previous study rat and human plasma were observed to inhibit the antagonist effect of [Leu\(^{11}\),d-Trp\(^{12}\)]rPTH(1-34) at the rat PTH1 receptor in osteosarcoma cells (Kukreja et al., 1994). In this study we examined the effect of human plasma on antagonism of the human PTH1 receptor expressed in COS-7 cells. At a concentration of 20%, human plasma had an antagonistic effect of human PTH1 receptor expressed in COS-7 cells (Kukreja et al., 1994). In this study we examined the rat model of HHM and in patients with HPT, suggesting that the antagonist is ineffective when the levels of PTH or PTHrP are high at the time of the antagonist infusion (Kukreja et al., 1994; Rosen et al., 1997). However, PTH1 receptor antagonists block the effects of administered PTH or PTHrP if the antagonist is infused before the agonist (Horiiuchi et al., 1983; Doppelt et al., 1986; Horiiuchi and Rosenblatt, 1987; Dresner-Pollak et al., 1996). We evaluated one component of the kinetics of antagonist action, the rate of ligand association to and dissociation from the PTH1 receptor. The indirectly determined dissociation rate constant of [d-Trp\(^{12}\),Tyr\(^{34}\)]bPTH(7-34) and PTHrP(7-34) was very high, implying rapid dissociation of these ligands from the PTH1 receptor (t\(_{1/2}\) values of 13 and 9 s, respectively). Rapid dissociation may contribute to the lack of efficacy of the ligand in the studies described above, in combination with the low plasma half-life (22 min for absorption of [Nle\(^{6,18}\),d-Trp\(^{12}\),Tyr\(^{34}\)]bPTH(7-34) and Schetz et al., 1995). The level of receptor occupancy predicted by an equilibrium model (used to calculate the doses used in the studies above) may not have been reached if the antagonist degrades rapidly, a problem that may be exacerbated if the antagonist dissociates rapidly from the receptor. The presence of high agonist levels before antagonist administration would enhance this effect, by slowing antagonist association with the receptor and exposing the ligand in the circulation for longer. The dissociation rate constant for TIP(7-39) was much lower (t\(_{1/2}\) value of 14 min). The slower dissociation of this antagonist may improve the level of receptor occupancy in vivo, increasing the antagonist effect. However the effectiveness of TIP(7-39) as an antagonist in vivo will probably be most dependent on the plasma half-life, which remains to be determined.

In conclusion, we have identified a novel PTH1 receptor antagonist, TIP(7-39), that displays a more favorable in vitro pharmacological profile than antagonists derived from the structures of PTH or PTHrP. Radiolabeled TIP(7-39) provides for the first time a labeled antagonist devoid of detectable agonism for use in radioligand binding studies. TIP(7-39) should prove useful for evaluating the effectiveness of PTH1 receptor antagonism in the reduction of elevated serum calcium levels. If this utility can be demonstrated, TIP(7-39), structurally modified analogs, or more stable low-molecular-weight PTH1 receptor antagonists may provide a new therapeutic strategy for the treatment of hypercalcemia.

Acknowledgment

We gratefully acknowledge Jon Marsh for patient assistance with the measurement of intracellular calcium.

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