New insights into interactions between the human PTH/PTHrP receptor and agonist/antagonist binding

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Fukayama, Shoichi, Miryam Royo, Masahiko Sugita, Amy Imrich, Michael Chorev, Larry J. Suva, Michael Rosenblatt, and Armen H. Tashjian, Jr. New insights into interactions between the human PTH/PTHrP receptor and agonist/antagonist binding. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E297–E303, 1998.—We prepared a polyclonal antiserum [Ab-(88—97)] against residues 88–97 of the NH2-terminal tail of the human (h) parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptor. Ab-(88—97) bound specifically to the receptor, as assessed by fluorescence-activated cell sorter analysis of HEK C21 cells, which stably express ~400,000 hPTH/PTHrP receptors per cell. Unlike PTH, Ab-(88—97) binding did not elicit either adenylate 3’,5’-cyclic monophosphate or intracellular calcium concentration signaling responses in these cells. Incubation of C21 cells for 90 min at 4°C with hPTH-(1—34) plus antiserum reduced the Ab-(88—97) binding to the cells by up to 40–50% of control values in a PTH concentration-dependent fashion with a half-maximal effective concentration of ~5 nM. The decrease in Ab-(88—97) binding caused by hPTH-(1—34) was completely reversed by coincubation with hPTHrP-(7—34). We conclude that residues 88—97 of the hPTH/PTHrP are involved, either directly or indirectly, in agonist but not antagonist binding to the receptor.

parathyroid hormone/parathyroid hormone-related protein receptor antibody; agonist; antagonist

PARATHYROID HORMONE (PTH) is the most important hormonal regulator of mineral ion homeostasis in mammals (21). PTH-related protein (PTHrP) was first identified and cloned from malignant tumor cells and tissues from patients with the syndrome of humoral hypercalcemia of malignancy (16, 25, 27). PTHrP-(1—34) and PTH-(1—34), which share a high degree of sequence identity in their NH2 terminus, act via a single species of cloned receptor (PTH/PTHrP) (1, 12, 21, 22, 23), although recent studies have shown that specific receptors for each of these peptides may also exist (29, 31).

Structure-function analyses of PTH and PTHrP peptides and the PTH/PTHrP have been studied extensively (4, 13, 14, 20). Although previous deletion, mutation, and chimeric investigations of the opossum, rat, and human PTH/PTHrP (13, 14) have suggested that the NH2-terminal extracellular tail of the PTH/PTHrP contains primary determinants for ligand binding, specific amino acid residues for the ligand-receptor interaction were not determined from these studies because alterations in the amino acid sequence of the NH2-terminal tail of the PTH/PTHrP often resulted in a dramatic decrease in the expression of mutant receptors on the cell surface (13, 14). However, these studies revealed that residues 61–105, encoded by exon E2 in the receptor gene, were not involved in ligand-receptor affinity (13, 14). More recently, by use of photoaffinity cross-linking, it has been shown that residues 173–189 of the human (h) PTH/PTHrP contain a ligand-receptor contact domain (32).

Antibodies directed against specific epitopes have been used in probing receptor structure and function in certain G protein-coupled receptors (3, 5, 28). In the process of studies on regulation of the hPTH/PTHrP (6), we have raised polyclonal antisera (Ab) against the NH2-terminal tail of the hPTH/PTHrP. In preliminary experiments in which we used one of these antisera [Ab-(88—97)] and HEK C21 cells (clone C21) stably expressing ~400,000 hPTH/PTHrP receptors/cell (18), we unexpectedly found that PTH-(1—34) attenuated the Ab-(88—97) binding. In this report, we show that PTH/PTHrP agonists (but not antagonists) decrease Ab-(88—97) binding to the receptor and that Ab-(88—97) also alters 125I-labeled PTH-(1—34) binding to the receptor.

MATERIALS AND METHODS

Materials. Culture media and sera were purchased from Gibco (Grand Island, NY), and tissue culture plasticware was obtained from Corning (Corning, NY). Transfection reagents, lipofectamine, and OPTI-MEM were purchased from Gibco-BRL (Grand Island, NY). Synthetic human PTH [hPTH-(1—34)NH2; lots ZM-080, ZL-216, and ZM-579]; human PTHrP [hPTHrP-(1—34)NH2; lots ZM-189 and SM-189]; hPTH-(53—84); lots 993C and ZG-027; hPTH-(44—68); lot QH-418; and hPTHrP-(107—139); lot ZJ-949; and hPTH-(3—34); lot 585D were purchased from Bachem California (Torrance, CA). Synthetic hPTH-(39—84); lot 027865 was from Peninsula (Belmont, CA). [Leu11,D-Trp12]hPTH-(7—34)NH2 was synthesized in the Division of Bone and Mineral Metabolism, Beth Israel Deaconess Medical Center, as described previously (12, 21). Fluorescein isothiocyanate (FITC)-conjugated swine antibodies to goat immunoglobulins were obtained from Biosource International (Camarillo, CA). All
other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Preparation of antibodies. After analysis of the hydrophilicity and antigenic indexes of the amino acid sequence of the cloned hPTH/PTHrPR (22, 23), as well as the species differences of the corresponding residues among other cloned PTH/PTHrP receptors (22), we selected amino acid residues 88–97 (NH2-terminus) and residues 477–497 (COOH-terminus) of the hPTH/PTHrPR as immunogens. A cysteine residue was introduced at one end of each peptide unless this residue was already present (as it was at position 98). These peptides were: 1) C-CTHR: [Cys488]hPTHrP-(478–498)NH2; 2) C-NPTHrP: hPTHrP-(89–98)NH2; 3) N-CTHR: Ac[Cys477]hPTHrP-(477–497)NH2; and 4) N-NPTHrP: Ac[Cys488]hPTHrP-(88–97)NH2. These peptides were then cross-linked to keyhole limpet hemocyanin via their terminal cysteine residues, as described (15). The conjugates were then sent to Berkeley Antibody (Richmond, CA) for custom antibody production. Mixtures of conjugates of peptides 1 plus 2 and 3 plus 4, in incomplete Freund's adjuvant, were each injected into goat 1 and goat 2, respectively, every 3 wk for eight immunizations in total. Serum samples were obtained 10 days after each immunization. Antibodies against the peptide sequences were monitored by fluorescence-activated cell sorter (FACS) analysis of use of either intact (for NH2-terminal receptor sequences) or permeabilized (for COOH-terminal sequences) HEK C21 cells (18).

Cell culture and transfection of DNA. Wild-type HEK 293 cells and HEK C21 cells stably expressing the cloned hPTH/PTHrPR were cultured as described previously (18).

Wild-type and mutant hPTH/PTHrPR DNAs (13, 22) were kindly donated by Drs. E. Schipani and H. Jüppner (Massachusetts General Hospital, Boston, MA). Subconfluent to confluent HEK 293 cells (lacking hPTH/PTHrPR), on 15-cm2 plastic tissue culture dishes, were transfected transiently by the lipofectamine method (according to the manufacturer's protocol). For FACS analysis with transiently transfected or untransfected cells, cells were harvested 72 h after transfection, as described below. For PTH/PTHrP binding assays with transiently transfected cells, cells were trypsinized and plated into 24-well plastic culture dishes 24 h after transfection. Forty-eight hours after plating (72 h after transfection), ligand binding assays were performed.

Ligand binding to the PTH/PTHrP. Confluent cells in 24-well plastic culture dishes were used for receptor binding experiments essentially as described previously (24, 30). Binding was determined by incubating cells with [125I]-labeled [Nle16,Tyr34]bovine PTH-(1–34)NH2 in the absence or presence of various concentrations of hPTH-(1–34) for 6 h at 4°C. Specific binding was determined as follows. Specific binding (%) = (total binding – nonspecific binding) × 100/(total tracer added – nonspecific binding), where nonspecific binding was determined in the presence of 10−6 M hPTH-(1–34).

Measurement of adenosine 3′,5′-cyclic monophosphate. Cells were treated with appropriate agonists (see RESULTS for experimental protocols) in the presence of 1 mM 3-isobutyl-1-methylxanthine, and the concentration of intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) in acid (50 mM HCl) extracts was measured by radiomunnoassay, as described previously (17).

FACS analysis. Cells grown on 15-cm2 plastic dishes were harvested as described previously (10) and aliquoted into 2.0-ml microtubes (1–3 million cells/tube). For permeabilization, cells were first fixed in 2% paraformaldehyde in N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)-buffered salt solution (Hank's balanced salt solution [HBSS] II, containing, in (mM): 118 NaCl, 4.6 KCl, 10 d-glucose, and 20 HEPES, pH 7.4) for 10 min at 4°C. Cells were then permeabilized with 0.1% saponin in HBSS II for 10 min at room temperature. After the washing with HBSS II, cells were incubated with test sera (1st Ab) at 4°C for 90 min unless otherwise specified. After a triple wash with HBSS II, cells were then incubated with a second antibody (FITC-conjugated swine antibodies to goat immunoglobulins) at 4°C for 90 min, unless otherwise specified, and cellular fluorescence was measured using either an Ortho 2150 cytofluorograph equipped with an air-cooled argon laser (488 nm excitation line, 15 mW output) or a FACSort flow cytometer (Becton-Dickinson, Mountain View, CA). Propidium iodide (PI) was added before flow analysis as a measure of cell viability. Live cells that were able to exclude PI were selected for determination of fluorescence.

RESULTS

Characterization of antibodies. Incubation with serum samples from goat 2 elicited a marked increase in fluorescence in intact HEK C21 cells but not in wild-type HEK cells (data not shown). The fluorescence intensity increased after each subsequent booster and reached a plateau after the sixth immunization. Such an increase in fluorescence was not observed when the cells were incubated with preimmune serum (data not shown). These results indicate that the immune serum samples recognize the NH2-terminal tail of the hPTH/PTHrPR (residues 88–97). Goat 2 was immunized with both hPTHrP-(477–497)NH2 and Ac[Cys488]hPTHrP-(88–97)NH2. To examine whether the antisera also contained antibody against COOH-terminal residues (477–497) of the hPTH/PTHrP, we permeabilized HEK C21 cells, as described in MATERIALS AND METHODS. Permeabilization of the cells did not increase the fluorescence further (data not shown), indicating that the antisera contains antibodies directed predominantly against the NH2-terminal tail of the hPTH/PTHrP. Significant antibody binding was observed in HEK cells transiently expressing the wild-type hPTH/PTHrP but not in cells transiently expressing a mutant hPTH/PTHrP in which the E2 domain (residues 61–105) was deleted (13) (Table 1). The expression level of the mutant receptor, as assessed by radiodilution binding, was ~70% that of the wild-type hPTH/PTHrP (Table 1). No significant fluorescence was observed when HEK BP16 cells, which stably express the PTH2 receptor (2), were incubated with the same antisera (data not shown). Moreover, fluorescence was blocked by 15 μg/ml of the peptide antigen N-NPTHrP but not by the C-CTHR peptide (Table 2). These results demonstrate that the antisera contains antibodies against NH2-terminal residues 88–97 of the human PTH/PTHrP. Therefore, we have used antisera from the eighth bleeding from goat 2 [Ab-(88–97)] in most of the subsequent experiments. Significant fluorescence was observed when the antisera was diluted as much as 1:25,600 (data not shown). We also found little or no difference in background fluorescence intensity when HEK C21 cells were incubated without (control) or with preimmune serum (data not shown). Therefore, because limited amounts of preimmune serum were available, cells incubated without the first...
Ab-(88–97) and PTH-(1–34) binding in PTH/PTHrP-transfected cells

Table 1. Ab-(88–97) and PTH-(1–34) binding in PTH/PTHrP-transfected cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>MFI</th>
<th>Specific 125I-PTH-(1–34) Binding, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transient transfection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.9 ± 0.5</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>hPTH/PTHrPR</td>
<td>6.6 ± 0.6</td>
<td>126 ± 15</td>
</tr>
<tr>
<td>E2 deletion</td>
<td>1.7 ± 0.4</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td>Stable transfection</td>
<td>6.9 ± 1.0</td>
<td>162 ± 17.2</td>
</tr>
</tbody>
</table>

Values are means ± SE for triplicate cultures. Similar results were obtained in 2 additional experiments of similar design. Wild-type HEK cells were transiently transfected without (control) or with cDNAs for wild-type human parathyroid hormone/PTH-related protein receptor (hPTH/PTHrP) or mutant hPTH/PTHrP in which the exon E2 domain (residues 61–105) was deleted. Ab-(88–97) and 125I-PTH-(1–34) binding was determined 72 h after transfection, as described in MATERIALS AND METHODS. A marked increase in mean fluorescence intensity (MFI) was observed in cells transfected with wild-type PTH/PTHrP but not in cells transfected with the E2 deletion mutant, even though the specific 125I-PTH-(1–34) binding was ~70% as high as that in cells transiently expressing the wild-type hPTH/PTHrP. HEK C21 cells stably express 400,000 hPTH/PTHrP receptors/cell (20). Specific 125I-PTH-(1–34) binding was determined as described in MATERIALS AND METHODS. Significantly different from MFI observed with antibody (Ab) alone: *P < 0.05; †P < 0.01.

Effects of PTH/PTHrP agonist/antagonist peptides on Ab-(88–97) binding. Incubation of C21 cells for 90 min at 4°C with 10−7 M hPTH-(1–34) plus Ab-(88–97) reduced Ab-(88–97) binding by up to 40–50% of control values (Table 3). When the cells were preincubated with 10−7 M hPTH-(1–34) for 15 min before addition of Ab-(88–97) at 4°C, somewhat more inhibition of Ab-(88–97) binding was observed (Table 4). When the cells were incubated with 10−7 M hPTH-(1–34) plus Ab-(88–97) at 37°C for 90 min, there was only a minimal decrease in Ab-(88–97) binding (Table 3). Moreover, Ab-(88–97) binding at 37°C was significantly lower than that at 4°C (Table 3). These results argue against the hypothesis that conformational change(s) in the receptor induced by hPTH-(1–34) plays a major role in the decrease in Ab-(88–97) binding.

Table 2. Specificity of the antiserum

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No 1st Ab</td>
<td>5.8 ± 0.90</td>
</tr>
<tr>
<td>Ab-(88–97)</td>
<td>342 ± 9.2</td>
</tr>
<tr>
<td>Ab-(88–97) + N-NPThr, 15 µg/ml</td>
<td>108 ± 5.2</td>
</tr>
<tr>
<td>Ab-(88–97) + C-CPTHR, 15 µg/ml</td>
<td>363 ± 9.8</td>
</tr>
<tr>
<td>Ab-(88–97) + N-NPRhr + C-CPTHR</td>
<td>114 ± 9.1</td>
</tr>
<tr>
<td>Ab-(88–97) + 10−8 M hPTH-(1–34)</td>
<td>252 ± 8.6*</td>
</tr>
<tr>
<td>Ab-(88–97) + 10−6 M [Leu11,D-Trp12]-hPTHrP-(7–34)</td>
<td>398 ± 9.8</td>
</tr>
<tr>
<td>Ab-(88–97) + 10−6 M hPTH-(1–34) + 10−6 M hPTHrP-(7–34)</td>
<td>382 ± 10.5</td>
</tr>
</tbody>
</table>

HEK C21 cells were incubated without (control) or with 1:100 dilution of Ab-(88–97) in the absence or presence of specific peptide immunogens, N-NPThr (15 µg/ml) or C-CPTHR (15 µg/ml), at 4°C for 90 min. Ab-(88–97) and peptides were mixed together at room temperature 30 min before incubation with cells. Each value gives mean MFI ± SE for triplicate cultures. Similar results were obtained in 2 additional experiments of similar design. When results from multiple experiments were analyzed, the increase in MFI observed with 10−6 M hPTHrP-(7–34) was not statistically significant (also see Table 4); however, the decrease in MFI observed with 10−8 M hPTH-(1–34) was significant (*P < 0.05).

Table 3. Effects of incubation of cells with Ab-(88–97) and PTH

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No 1st Ab</td>
<td>7.0 ± 0.7</td>
</tr>
<tr>
<td>Ab-(88–97)</td>
<td>353 ± 4.3</td>
</tr>
<tr>
<td>Ab-(88–97) + 10−7 M hPTH-(1–34)</td>
<td>220 ± 3.5c</td>
</tr>
<tr>
<td>Ab-(88–97) + 10−7 M hPTH-(1–34)</td>
<td>202 ± 5.0d</td>
</tr>
<tr>
<td>Ab-(88–97) + 10−7 M hPTH-(1–34)</td>
<td>167 ± 4.9e</td>
</tr>
</tbody>
</table>

HEK C21 cells were incubated without or with Ab-(88–97) (1:100) for 90 min at 4°C or 37°C. Each values gives mean ± SE for triplicate cultures. Similar results were obtained in 2 additional experiments of similar design. *P < 0.01, †P < 0.05. Significantly different from no preincubation, ‡P < 0.05.

Effects of various PTH/PTHrP peptides on Ab-(88–97) binding

Table 4. Effects of various PTH/PTHrP peptides on Ab-(88–97) binding

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No 1st Ab</td>
<td>9.5 ± 0.4</td>
</tr>
<tr>
<td>Ab-(88–97)</td>
<td>275 ± 17</td>
</tr>
<tr>
<td>Ab-(88–97) + hPTH-(1–34)</td>
<td>142 ± 16*</td>
</tr>
<tr>
<td>Ab-(88–97) + hPTHrP-(1–34)</td>
<td>164 ± 8*</td>
</tr>
<tr>
<td>Ab-(88–97) + bPTH-(3–34)</td>
<td>278 ± 37</td>
</tr>
<tr>
<td>Ab-(88–97) + [Leu11,D-Trp12]-hPTHrP-(7–34)</td>
<td>280 ± 12</td>
</tr>
<tr>
<td>Ab-(88–97) + hPTHrP-(107–139)</td>
<td>301 ± 29</td>
</tr>
<tr>
<td>Ab-(88–97) + hPTH-(39–84)</td>
<td>262 ± 28</td>
</tr>
<tr>
<td>Ab-(88–97) + hPTH-(53–84)</td>
<td>269 ± 2</td>
</tr>
</tbody>
</table>

HEK C21 cells were incubated with Ab-(88–97) (1:100) and various peptide fragments of the PTH/PTHrP (10−6 M) at 4°C for 90 min. After washing, cells were incubated with 2nd Ab (1:100) at 4°C for 90 min as described in MATERIALS AND METHODS. Each value gives mean ± SE for triplicate cultures. Similar results were obtained in 2 additional experiments of similar design. *Significantly different from control (P < 0.01).
Inhibition of Ab-(88–97) binding by coincubation with hPTH-(1–34) was observed in a concentration-dependent fashion with a half-maximally effective concentration (EC50) of 4–5 nM (Fig. 1). It has been shown that NH2-terminal PTH-(1–34) and PTHrP-(1–34) bind to and activate the PTH/PTHrPR in an indistinguishable manner in various experimental systems, including in HEK C21 cells (18, 19). Therefore, we examined the effect of hPTHrP-(1–34) on Ab-(88–97) binding. Unexpectedly, we found that significantly more hPTHrP-(1–34) than hPTH-(1–34) was required to inhibit Ab-(88–97) binding (EC50 of 15–20 nM; Fig. 1).

Incubation of HEK C21 cells with antisem plus 10–8 M [Leu11,D-Trp12]hPTHrP-(7–34), a potent antagonist of the PTH/PTHrPR (4), for 90 min at 4°C did not alter Ab-(88–97) binding (Tables 2 and 4). Likewise, coincubation with 10–6 M bovine PTH-(3–34) [bPTH-(3–34)], another antagonist of the PTH/PTHrPR, was also without effect on Ab-(88–97) binding (Table 4). Incubations of HEK C21 cells with 10–6 M hPTH-(39–84), hPTH-(53–84), or hPTHrP-(107–139), none of which bind to or activate the hPTH/PTHrPR (18, 19), were without effect on Ab-(88–97) binding (Table 4), arguing against nonspecific interactions between PTH/PTHrP peptides and the Ab-(88–97) or cells.

It has been shown in various cell systems that HEK C21 cells that [Leu11,D-Trp12]hPTHrP-(7–34) is a potent, specific antagonist of the hPTH/PTHrPR and blocks the cAMP/protein kinase A (PKA) and Ca2+/protein kinase C signaling pathways elicited by hPTH-(1–34) or hPTHrP-(1–34) (2, 11, 17). Therefore, we examined the effect of [Leu11,D-Trp12]hPTHrP-(7–34) on the effect of hPTH-(1–34). The decrease in Ab-(88–97) binding elicited by 10–8 M hPTH-(1–34) was completely reversed by coincubation with 10–6 M [Leu11,D-Trp12]hPTHrP-(7–34) (Table 2), consistent with the conclusion that inhibition of Ab-(88–97) binding by hPTH-(1–34) is mediated via agonist binding to the hPTH/PTHrPR.

Effects of Ab-(88–97) on PTH binding. We next examined whether incubation of HEK C21 cells with Ab-(88–97) inhibited 125I-hPTH-(1–34) binding. Incubation of HEK C21 cells with Ab-(88–97) plus 125I-PTH-(1–34) for 6 h at 4°C reduced labeled tracer binding by up to 30% without changing affinity (~1–3 nM). Inhibition of 125I-PTH-(1–34) binding by Ab-(88–97) was blocked by preincubation of the antisem with the specific peptide antigen (data not shown).

Effects of Ab-(88–97) on signal transduction pathways mediated via the PTH/PTHrPR. Because our data suggest that the recognition epitope(s) of Ab-(88–97) might overlap with the agonist-receptor interaction domain(s) on the PTH/PTHrPR, we examined whether Ab-(88–97) alone could stimulate cAMP production or whether Ab-(88–97) could block PTH-stimulated cAMP production. As shown in Fig. 2, Ab-(88–97) did not stimulate cAMP production, nor did it block cAMP production stimulated by 10–8 M hPTH-(1–34). Similarly, addition of 1:100 Ab-(88–97) did not induce intracellular calcium concentration ([Ca2+]i) transients, nor did it block the rise in [Ca2+]i stimulated by 10–8 M hPTH-(1–34) in HEK C21 cells (data not shown).

However, prolonged pretreatment (≥12 h) of HEK C21 cells with Ab-(88–97) (1:400) at 37°C significantly decreased subsequent cAMP production stimulated by 10–8 M hPTH-(1–34) to 40–50% of control values, whereas pretreatment with 10–7 M hPTH-(1–34) for 12 h at 37°C induced homologous cAMP desensitization to 20–30% of control (Fig. 3). As reported previously (9), pretreatment with hPTH-(1–34) dramatically enhanced the subsequent cAMP response to 10–5 M forskolin (Fsk) (Fig. 3). Preincubation with Ab-(88–97)
METHODS. Values are means $\pm$ SE for 4 cultures. Similar results were obtained in 2 additional experiments of similar design. Dashed horizontal line, cellular cAMP concentration in response to $10^{-8}$ M hPTH-(1—34) in control cells. *P $< 0.05$ and **P $< 0.01$ vs. pretreatment control values.

was without effect on Fsk-stimulated cAMP production (Fig. 3), indicating that the action of Ab-(88—97) was mediated via the PTH/PTHrPR. Preincubation with preimmune serum had no effect on cAMP production stimulated by either $10^{-8}$ M hPTH-(1—34) or $10^{-5}$ M Fsk (Fig. 3).

DISCUSSION

Previous analysis of opossum, rat, and human PTH/PTHrPRs transiently expressed in COS cells has revealed that deletion of residues 61–105, encoded by exon E2 in the receptor gene, and insertion of an epitope derived from Haemophilus influenza hemagglutinin into that sequence did not affect the receptor function, as assessed by ligand-receptor affinity and cAMP production (13, 14). Deletion of amino acids of the NH$_2$-terminal tail of rat or opossum PTHrPR receptors encoded by exon 1 (r$\Delta E1$, residues 26–60), exon 3 (r$\Delta E3$, residues 106–141), or exon G (r$\Delta G$, residues 142–181) resulted in almost complete loss of surface expression and radioligand binding as assessed by use of antibody to the HA-tagged receptor, whereas deletion of amino acids encoded by exon 2 (r$\Delta E2$, residues 61–105) did not alter ligand-receptor affinity (14). Similarly, deletion of amino acids corresponding to exon 2 of the PTHrPR receptors did not affect ligand-receptor affinity when transiently expressed in COS cells (13). Moreover, amino acid sequences of exon 2-corresponding residues of PTHrPRs are not well conserved among species from which PTHrPRs have been cloned (22). On the basis of these findings, it has been concluded that the exon 2-corresponding domain of PTHrPRs is not involved in ligand-receptor binding (13, 14), although these studies did not determine the number of receptors expressed on the cell surface. More recently, photoaffinity cross-linking studies have directly identified residues 173–189 of the hPTHrPR receptor as the contact domain to the amino acid residue in position 13 of a bioactive PTH-(1—34)-derived ligand (32). In that study, a recombinant hPTHrPR receptor stably expressed in HEK C21 cells (~400,000 receptors/cell) was photoconjugated to a radioactive, photoactive, bioactive ligand, [Nle$^{8,18}$,Lys$^{13}$(e-3-l-PBz)$_2$,2-Nal$^{23}$,Arg$^{26,27}$,Tyr$^{34}$]PTH-(1—34)NH$_2$. This 2113-Da cross-linked domain, located at the distal end of the NH$_2$-terminal tail of the PTHrPR receptor, is directly involved in ligand-receptor interaction.

Antibodies against specific epitopes have been used in probing the structure and function of several membrane receptors (3, 5, 28). For example, by use of antibodies against the cloned glucagon receptor, it has been proposed that the principal ligand binding/activation domains of the receptor are located in residues 126–137 of the NH$_2$-terminal tail and residues 206–219 in the first extracellular loop (28).

In the present study, we have prepared an antiserum that recognizes amino acid residues 88–97 of the NH$_2$-terminal tail of the hPTH/PTHrPR. Using Ab-(88—97), we have shown that incubation of HEK C21 cells with this antiserum plus hPTH-(1—34) reduced Ab-(88—97) binding to the cells, and that Ab-(88—97) also reduced $^{125}$I-hPTH-(1—34) binding. Interestingly, inhibition of Ab-(88—97) binding was not observed when the cells were incubated with $10^{-6}$ M [Leu$^{14}$,D-Trp$^{12}$]hPTHrPR-(7—34) or bPTH-(3—34), two PTH/PTHrP antagonists. Because inhibition of Ab-(88—97) binding by $10^{-8}$ M hPTH-(1—34) was reversed completely by coincubation with $10^{-6}$ M [Leu$^{14}$,D-Trp$^{12}$]hPTHrPR-(7—34), the decrease in Ab-(88—97) binding induced by hPTH-(1—34) appears to be mediated via the PTHrPR. These findings can be interpreted in at least two ways.

First, the recognition epitope of Ab-(88—97) may overlap with the receptor’s hormone binding domain(s), suggesting that residues 88—97 in the receptor contain agonist, but not antagonist interaction sites; thus Ab-(88—97) and PTH/PTHrP-(1—34) agonists partially compete for receptor binding sites. It should be noted, however, that PTH/PTHrP agonists inhibited Ab-(88—97) binding only modestly (~40–50%), suggesting that residues 88—97 of the hPTH/PTHrP contribute, if at all, only to low-affinity interaction site(s). Inhibition of $^{125}$I-hPTH-(1—34) binding by Ab-(88—97) could be explained by direct competition between agonists and Ab-(88—97) within residues 88—97 of the NH$_2$-terminal tail.

Alternatively, it is possible that the binding of agonist, but not antagonist, alters the conformation of the 88—97 domain of the hPTH/PTHrP to result secondarily in a decrease in Ab-(88—97) binding. In this case, inhibition of $^{125}$I-hPTH-(1—34) binding can be explained by secondary or steric effects of the antibody at a distance on agonist binding domain(s). However, antibody- or agonist-induced conformational changes of the
hPTH/PTHrPR are considered to be diminished because our experiments were performed at 4°C, where physiological conformational changes are less likely to occur (4). Additional evidence against a major contribution of conformational changes in the hPTH/PTHrPR to the inhibition of Ab-(88–97) binding is that addition of hPTH-(1–34) before the incubation with Ab-(88–97) at 4°C resulted in increased inhibition of Ab-(88–97) binding greater than when hPTH-(1–34) and Ab-(88–97) were added together. Moreover, only a minimal, if any, decrease in Ab-(88–97) binding was observed at 37°C, a temperature at which conformational changes of the hPTH/PTHrPR would be more likely. Inhibition of Ab-(88–97) binding by PTH at 37°C could be explained by internalization of the PTH/PTHrPR after PTH (and possibly Ab) binding to the receptor. Unlike previous findings with thyroid-stimulating hormone (5) and glucagon (3, 28), receptors, in which specific receptor antibodies blocked both ligand binding and agonist-stimulated cAMP production, we found no inhibition by Ab-(88–97) of cAMP production stimulated by hPTH-(1–34), suggesting that residues 88–97 of the hPTH/PTHrPR play little or no role in activating the cAMP signaling pathway by hPTH-(1–34). However, we have shown that prolonged (≥12-h) preincubation with Ab-(88–97) induced a significant decrease in the cAMP response to subsequent stimulation with hPTH-(1–34). Because Ab-(88–97) alone did not stimulate cAMP production in HEK C21 cells, Ab-(88–97)-induced cAMP desensitization of the PTH/PTHrPR would appear to be mediated via a cAMP-independent mechanism. It has been shown that PTH-induced homologous cAMP desensitization involves both cAMP/PKA-dependent and -independent mechanisms (8, 9). More recently, we have shown that activation of β-adrenergic receptor kinase-1 is a critical component of the acute phase (≤2 h) of PTH-induced homologous downregulation and desensitization of the PTH/PTHrPR in human osteoblast-like SaOS-2 cells (6). It is not likely, however, that analogous mechanisms are involved in Ab-(88–97)-induced cAMP desensitization of the PTH/PTHrPR because acute (≥1-h) preincubation with Ab-(88–97) was without effect on subsequent cAMP response to PTH. One possible explanation is that prolonged preincubation with Ab-(88–97) induces downregulation of the PTH/PTHrPR, which, in turn, results in a decrease in the cAMP response to PTH.

In the present study, we found that hPTHrP-(1–34) is less potent than hPTH-(1–34) in reducing Ab-(88–97) binding. It has been accepted that NH2-terminal PTHrP-(1–34) and PTH-(1–34) can bind to and activate the PTH/PTHrPR in an indistinguishable manner (12, 18, 21). This is also the finding for HEK C21 cells that stably express the cloned hPTH/PTHrPR (2, 18). Taken together, our findings suggest subtle differences between PTH-(1–34) and PTHrP-(1–34) in the mode of binding to and/or activating the common receptor.

Finally, in the present study we found that PTH/PTHrP antagonists such as hPTHrP-(7–34) or bPTH-(3–34) do not inhibit Ab-(88–97) binding, results consistent with previous findings that deletion of the E2-corresponding domain (residues 61–105) of the hPTH/PTHrPR did not alter the apparent PTH-(7–34) binding affinity (13); however, we have shown that PTH/PTHrP agonists such as hPTH-(1–34) or hPTHrP-(1–34) reduce Ab-(88–97) binding. As discussed above, we do not know whether the different effects induced by PTH/PTHrP agonists and antagonists are due to differences in their binding characteristics to the receptor or to induced conformational changes of the PTH/PTHrPR.

We are grateful to Drs. Sunil Shaw and Chi-Chang Shieh (Lymphocyte Biology Section, Div. of Rheumatology and Immunology, Brigham and Women’s Hospital) for help in the choice of antigenic peptides and technical assistance in the initial phase of antibody characterization by FACS analysis, respectively. We also thank Jean Foley for excellent assistance in the preparation of this manuscript.

This investigation was supported in part by research grants from the National Institute of Diabetes, Digestive and Kidney Diseases (DK-46655 and DK-10206 to A. H. Tashjian, Jr. and DK-47940 to M. Rosenblatt).

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Received 4 September 1997; accepted in final form 10 November 1997.

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