Polar residues within the transmembrane domains (TMs) of G protein-coupled receptors have been implicated to be important determinants of receptor function. We have identified mutations at two polar sites in the TM regions of the rat parathyroid hormone (PTH)/PTH-related peptide receptor, Arg-233 in TM 2 and Gin-451 in TM 7, that caused 17-200-fold reductions in the binding affinity of the agonist peptide PTH-(1–34) without affecting the binding affinity of the antagonist/partial agonist PTH-(3–34). When mutations at the TM 2 and TM 7 sites were combined, binding affinity for PTH-(1–34) was restored to nearly that of the wild-type receptor. The double mutant receptors, however, were completely defective in signaling cAMP or inositol phosphate production in response to PTH-(1–34) agonist ligand. The results demonstrate that Arg-233 and Gin-451 have important roles in determining agonist binding affinity and transmembrane signaling. Furthermore, the finding that residues in TM 2 and TM 7 are functionally linked suggests that the TM domain topology of the PTH/PTHrP-related peptide receptor may resemble that of receptors in the rhodopsin/β-adrenergic receptor family, for which structural and mutagenesis data suggest interactions between TMs 2 and 7.

The PTHrP receptor (PPR) is a member of a distinct G protein-coupled receptor family that includes the receptors for secretin, calcitonin, glucagon, and several other unmodified peptide hormones of intermediate size (25–60 amino acids) (1, 2). The receptors in this group display no apparent sequence homology to receptors in the larger G protein-coupled receptor family. TM domains 2 and 7 are close to each other and could, therefore, interact. Recent mutagenesis data on several members of the β-adrenergic receptor family support the notion that residues in transmembrane domains 2 and 7 of these G protein-coupled receptors interact (12, 20–23). The TM domains of the PTH/PTHrP receptor contain a number of polar residues that could likewise be important for receptor structure and/or function. In the course of evaluating some of these residues in the rat PPR, we observed that mutations at two sites, Arg-233 in TM 2 or Gin-451 in TM 7 had marked effects on ligand binding. In this paper we show that point mutations at either site markedly impair the binding of the agonist, PTH-(1–34), without affecting the binding of the antagonist PTH-(3–34). Furthermore, when the mutations at the two sites are combined, then agonist binding affinity is restored, although signal transduction capability is severely impaired. The data suggest that residues in TM 2 and TM 7 of the PTHrP receptor are functionally linked and that this putative interaction is important for transmission of the hormone’s signal across the cell membrane.

EXPERIMENTAL PROCEDURES

Peptides and Reagents—[Nle8,18,Tyr34]bPTH-(3–34)NH2, [Thr34]hPTH-(3–34)NH2, [Tyr34]hPTH-(1–34), and [Tyr34]hPTH-(3–34)NH2 were prepared by solid phase synthesis on an Applied Biosystems model 430A peptide synthesizer using Fmoc (9-fluorenylmethoxycarbonyl) chemistry and purified by HPLC. Na[125I] (2,000 Ci/mmol), 125I-labeled goat anti-mouse IgG antibody, and [3H]myo-inositol (20.5 Ci/mmol) were purchased from DuPont NEN. The anti-PA monoclonal antibody, 12CAS (24), was purchased from Boehringer Mannheim. Oligonucleotides used for mutagenesis and DNA sequence analysis were constructed on an Applied Biosystems model 380A DNA synthesizer. DNA modifying enzymes were from U. S. Biochemical Corp.

Receptor Mutagenesis—Mutations were introduced into the rWT-HA
cDNA sequence carried on the pCDNA-1 plasmid vector. The rWT-HA DNA sequence encodes the rat PPR containing the HA (human influenza virus hemagglutinin) epitope tag in the extracellular E2 region (4). Point mutations were incorporated into rWT-HA single-stranded plasmid DNA by oligonucleotide-directed mutagenesis (25). The mutagenesis procedure was random with a bias that favored conservative polar substitutions; thus the codon corresponding to Arg-233 (CGC) was changed to (A/C)A(A/C), (His, Gln, Asn, and Lys), and the codon corresponding to Gln-451 (CAG) was changed to (A/G)A(G/C) (Asn, Lys, Asp, and Glu), where the slash denotes an equal mixture of two nucleotides. Mutations at the TM2 and TM7 sites were combined by exchanging the appropriate ApaLI-ApaLI restriction enzyme DNA fragments. Mutations were verified by nucleotide sequence analysis of single-stranded plasmid DNA.

Cell Culture and DNA Transfection—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah), 20 units/ml penicillin G, 20 μg/ml streptomycin sulfate, and 0.05 μg/ml amphotericin B in a humidified atmosphere containing 95% air/5% CO₂. Transfections were performed with plasmid DNA that had been purified on cesium chloride gradients. Cells were seeded in 24-well plates (50,000 cells/well) and transfected (100 ng DNA/well) by the DEAE/dextran method (26) when the monolayer reached 90–95% of confluence. Assays were performed on the intact transfected COS-7 cells 72 h after transfection, at which point the cell density was ~550,000 cells/well.

Antibody Binding to Epitope-tagged Receptors—The use of PPRs containing the HA epitope tag in a nonessential extracellular region to assess receptor surface expression has been described previously (4). Briefly, transfected COS-7 cells were rinsed with binding buffer (50 mM Tris-HCl, pH 7.7, 100 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 5% heat-inactivated horse serum, 0.5% fetal bovine serum), and 250 μl of binding buffer containing the monoclonal antibody 12CA5 (1.25 μg/well) was added. After 2 h at 15°C, the cells were rinsed with binding buffer, and 250 μl of binding buffer containing ¹²⁵I-labeled goat anti-mouse IgG was added (~350,000 cpm/well). After 2 h at 15°C, the cells were washed with binding buffer and lysed with 0.5 ml of 50 mM NaOH, and the entire lysate was counted. Nonspecific binding was determined with mock-transfected cells and was 1,100 ± 100 cpm. Maximum specific binding to cells expressing rWT-HA was 1.2 ± 0.1% of total radioactivity added (n = 17). The radioactivity specifically bound by each mutant receptor was divided by the radioactivity specifically bound to the rWT-HA control (defined as 100%) included in each experiment and multiplied by 100.

Radioligand-Receptor Binding—PTH binding assays were performed as described previously (27). Radiolabeled bPTH-(3–34) analog was prepared by chloramine-T iodination and HPLC purification. Binding reactions were performed in 24-well plates (final volume, 300 μl/well) and contained 26 fmol of ¹²⁵I-bPTH-(3–34) (100,000 cpm) and successive doses (0.4–300 pmol) of an unlabeled competitor ligand and were incubated for 4 h at 15°C. After washing with binding buffer, the cells were lysed with NaOH and counted for radioactivity. Nonspecific binding, determined in wells containing mock transfected COS-7 cells, was ~1%.

![Fig. 1. Schematic of a portion of the PPR and alignment of transmembrane domains 2 and 7. The schematic at the top shows a possible map of the seven-membrane embedded helices of the rat PPR and the interconnecting loops; predictions for the TM and loop domains were guided by the PHDhtm PredictProtein algorithm (31). Arg-233 and Gin-451 are shown as black circles. At the bottom is shown an alignment of the regions predicted to contain TM domains 2 and 7 from several members of the PTH/secretin/calcitonin receptor family. The residues corresponding to Arg-233 and Gin-451 of the rat PPR are marked by asterisks. The alignment was constructed using the full-length receptor sequences and the Pileup program of the Genetics Computer Group (G.C.G., Madison, WI) software package. Receptor segments shown are: rPPR, rat PTH/PTHrP-R (2); rGRFR, growth hormone-releasing factor-R (34); rPACAPR, pituitary adenyl cyclase-activating peptide-R, type I (35); rSecR, secretin-R (36); rVIP2R, vasoactive intestinal peptide-R type 2 (37); rVIPR, vasoactive intestinal peptide-R type 1 (38); rGlucR, glucagon-R (40); rGLP1R, glucagon-like peptide 1-R (41); hCRFRA, human corticotropin-releasing factor-R type A (42); M.s. dhR, Manduca sexta diuretic hormone-R (43); and rCTR, calcitonin-R (44).](http://www.jbc.org/content/12821/3/12821/F1.large.jpg)
Transmembrane Residues in the PTH/PTHrP Receptor

Table 1

Expression of WT and mutant PTH/PTHrP receptors

Intact COS-7 cells transfected with plasmid DNA (100 ng/well) encoding rWT-HA or a mutant PPR, each containing an extracellular epitope tag (HA) were assessed for maximum specific binding of antibody (12CA5 plus 125I-labeled anti-mouse IgG), 125I-bPTH-(1–34) (125I-[Ni613,Tyr16]bPTH-(1–34)NH2) and 125I-bPTH-(3–34) (125I-[Ni613,Tyr34]bPTH-(3–34)NH2) to rWT-HA and each mutant receptor. Maximum specific binding (Bmax) was determined from plots of log(competitor) versus binding (shaded area) and varied from 300 pmol to 1000 pmol per well. The number of binding sites per cell was determined by Scatchard analysis performed with 125I-bPTH-(3–34) binding sites per cell was derived from Scatchard analysis performed with 125I-bPTH-(3–34) analog (100,000 cpm) and varying concentrations (3–1,000 nM) of unlabeled peptide. Data are the mean (±S.E.) of the number of independent experiments indicated in parentheses, each performed in duplicate or triplicate.

| PPR     | Antibody          | 125I-bPTH-(1–34) analog | 125I-bPTH-(3–34) analog | bPTH-(3–34) binding sites/cell |
|---------|-------------------|-------------------------|-------------------------|-------------------------------|
|         | % of rWT-HA       | % of total              | % of total              |                               |
| rWT-HA  | 100 ± 1 (17)      | 17.6 ± 1.15             | 29.5 ± 1.4 (14)         | 2.5 ± 0.4 (8)                 |
| R233H   | 108 ± 5 (17)      | 6.6 ± 0.7 (12)          | 29.7 ± 2.6 (11)         | 3.3 ± 0.4 (7)                 |
| R233Q   | 93 ± 3 (14)       | 17.7 ± 1.7 (8)          | 36.9 ± 3.3 (8)          | 2.0 ± 0.4 (4)                 |
| R233N   | 86 ± 3 (12)       | 13.6 ± 1.6 (7)          | 28.0 ± 3.2 (7)          | 1.7 ± 0.1 (2)                 |
| R233K   | 112 ± 4 (12)      | 20.1 ± 7 (17)           | 41.0 ± 3.4 (7)          | 2.5 ± 0.4 (3)                 |
| Q451K   | 56 ± 3 (13)       | 2.3 ± 0.3 (13)          | 19.1 ± 1.5 (12)         | 1.2 ± 0.3 (8)                 |
| R233H/Q451K | 82 ± 3 (4)     | 23.2 ± 1.9 (5)          | 38.9 ± 1.7 (7)          | 3.6 ± 1.1 (3)                 |
| R233Q/Q451K | 87 ± 4 (4)     | 23.1 ± 2.3 (5)          | 39.5 ± 2.1 (7)          | 4.7 ± 0.9 (3)                 |
| R233N/Q451K | 99 ± 5 (2)     | 17.5 ± 2.5 (3)          | 35.6 ± 3.1 (5)          | 4.3 ± 0.8 (3)                 |

Fig. 2. PTH binding properties of WT and mutant PPRs. Intact COS-7 cells transiently transfected with the WT or the indicated mutant PPR were evaluated in competition binding studies; 125I-[Ni613,Tyr16]bPTH-(1–34)NH2 (100,000 cpm/well) was used as a radioligand and [Try16]bPTH-(1–34)NH2 (50 pmol/well) was used as an unlabeled competitor ligand. Each curve shows the mean (± S.E.) of data compiled from 5–10 experiments, each performed in duplicate.

Cyclic AMP Production—24-well plates containing transfected cells were chilled on ice, rinsed with 500 μl of binding buffer, and treated with 200 μl of ice-cold cAMP assay buffer (Dulbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine, 1 mg/ml bovine serum albumin, 35 mM Hapes-NaOH, pH 7.4) and 100 μl of binding buffer containing various concentrations of PTH ligand. After 30 min at 37 °C, the buffer was removed, and the cells were lysed by placing the plates on powdered dry ice and adding 0.5 ml of 50 mM HCl. The cAMP content of a 5–50-μl aliquot of the diluted lysate (1:6 with H2O) was determined by radioimmunoassay.

Inositol Phosphate Production—48 h after transfection, cell medium was replaced by 0.5 ml of inositol-free Dulbecco's modified Eagle's medium containing [3H]myo-inositol (2 μCi/ml), and incubation continued overnight at 37 °C. Cells were washed with 0.5 ml of binding buffer containing LiCl2 (30 mM), and then 300 μl of binding buffer-LiCl2 with or without PTH ligand was added, and the cells were incubated for 30 min at 37 °C for 40 min. The buffer was then removed and replaced by 0.5 ml of ice-cold trichloroacetic acid (5%). After 1 h on ice, the acid lysate was extracted twice with ether and then applied to a column packed with 0.5 ml of Dowex AG 1-X8 resin (Bio-Rad) in 10 mM myo-inositol. The resin was washed with 10 ml of 10 mM moyo-inositol followed by 10 ml of 5 mM Borax/60 mM ammonium formate; the total inositol phosphates were eluted in 3 ml of 1 N ammonium formate/100 mM formic acid (28) and quantified by scintillation counting.

RESULTS

Fig. 1 shows the location of Arg-233 and Gln-451 in TMs 2 and 7 of the rat PPR and the conservation of these residues in the PTH/secretin/calcitonin receptor family. These two residues were targeted for analysis by random mutagenesis. To avoid possible impairment of receptor expression that might occur with nonconservative changes, the substitutions introduced at these positions were limited to other polar residues.

The effects of the resulting mutations at Arg-233 (Lys, Asn, and His) and Gln-451 (Lys) on receptor surface expression in transiently transfected COS-7 cells are reported in Table I. The WT PPR (rWT-HA) and each mutant receptor contained the phenotypically silent HA epitope tag in its amino-terminal extracellular domain. Mutant receptors with changes at Arg-233 bound anti-PTH antibody to levels that were at least 80% of those bound by the rWT-HA, whereas the Q451K mutant displayed a mild expression defect (56% of rWT-HA). Scatchard analysis of homologous competition binding studies performed with labeled and unlabeled [Ni613,Tyr34]bPTH-(3–34)NH2 confirmed that each of the mutant receptors was adequately expressed on the cell surface (Table I).

The maximum specific binding of the radiolabeled agonist peptide, 125I-bPTH-(1–34) analog to the R223H or Q451K mutant receptor (6.6 and 2.3% of total added, respectively) was markedly reduced from the corresponding value seen for rWT-HA (17.6%). The Asn substitution at Arg-233 caused a more modest decrease in 125I-bPTH-(1–34) binding (13.6), whereas the Lys and Gln substitutions at this site...
indicated that mutations at positions 233 and 451 differentially affected the binding of PTH-(1–34) and PTH-(3–34) analogs.

The binding of PTH-(1–34) and PTH-(3–34) analogs to the mutant receptors were compared further in competition binding studies. Fig. 2A shows that with the WT PPR, hPTH-(1–34) ([Tyr34]hPTH-(1–34)NH2) was 2–3-fold more potent than hPTH-(3–34) ([Tyr34]hPTH-(3–34)NH2) in inhibiting the binding of 125I-bPTH-(3–34) (IC50 values = 6.6 and 17 nM, respectively). With the R223H mutant receptor, the binding affinity of hPTH-(3–34)NH2 (IC50 = 19 nM) was comparable with rWT-HA (Fig. 2B and Table II), whereas in contrast there was a pronounced (190-fold) shift to the right in the binding potency of hPTH-(1–34) (IC50 = 1, 230). A similar shift to the right in the binding profile of the hPTH-(1–34) peptide was observed with the Q451K, R233Q, and R233N mutant receptors (Fig. 2C and Table II). Competition binding studies performed with bovine PTH analogs, [Nle8,18,Tyr34]bPTH-(1–34)NH2 and [Nle8,18,Tyr34]bPTH-(3–34)NH2 showed similar disproportional effects of the mutations on binding, although the binding of the bovine PTH-(1–34) analog was less severely impaired (Table II).

We then investigated the effects of combining the mutations at Arg-233 and Gin-451. As shown in Fig. 2D, the R233H/Q451K double mutant receptor bound hPTH-(1–34) with higher affinity (IC50 = 50 nM) than the corresponding single mutant receptors (Table II). Similar increases in binding affinity occurred when the Q451K change was combined with the R233Q or R233N mutations (Table II). Thus, combining mutations in TM 2 and TM 7 did not lead to additive reductions in PTH-(1–34) binding affinity, but instead a substantial rescue of the binding defects caused by the respective individual point mutations.

The transmembrane domains of G protein-coupled receptors and the intracellular connecting loops are likely to play important roles in signal transduction and G protein coupling. The effects of point mutations in TM 2 and TM 7 on the receptor’s ability to stimulate cAMP production in response to PTH ligands are shown in Fig. 3. COS-7 cells expressing rWT-HA responded to hPTH-(1–34) with a 20-fold increase in intracellular CAMP, as compared with the basal level of cAMP measured in the untreated cells (EC50 = ~0.4 nM; Fig. 3). With the exception of the R233K mutant, which exhibited a cAMP response equivalent to that of rWT-HA, the responses of the mutant receptors were considerably weaker than those of rWT-HA. The low responses precluded the definition of precise response maxima and EC50 values, although qualitative differences in the responses were consistently observed (Fig. 3). The R223H response curve was parallel and reached the maximum response of the WT PPR but was displaced 100-fold to the right. The Q451K, R233Q, and R233N mutants exhibited shallower responses reaching less than 50% of the WT maximum, and affected the binding of PTH-(1–34) and PTH-(3–34) analogs.

**TABLE II**

Ligand binding properties of WT and mutant PTH/PTHrP receptors

| PPR       | Binding affinity of competitor ligand (IC50) |
|-----------|---------------------------------------------|
|           | [Tyr34]hPTH-(1–34)NH2 | [Tyr34]hPTH-(3–34)NH2 | [Nle8,18,Tyr34]bPTH-(1–34)NH2 | [Nle8,18,Tyr34]bPTH-(3–34)NH2 |
| rWT-HA    | 6.6 ± 0.9 (14)        | 17 ± 2.0 (13)         | 7.2 ± 1.5 (8)                  | 10 ± 2 (9)                   |
| R233H     | 1,230 ± 170 (12)      | 19 ± 3 (10)           | 118 ± 16 (5)                   | 10 ± 2 (4)                   |
| R233Q     | 1,120 ± 240 (8)       | 27 ± 5 (6)            | 33 ± 3 (5)                     | 6.3 ± 0.9 (4)                |
| R233N     | 292 ± 36 (8)          | 14 ± 2 (8)            | 21 ± 2 (4)                     | 6.3 ± 0.7 (3)                |
| R233K     | 35 ± 5 (6)            | 21 ± 4 (4)            | 20 ± 3 (4)                     | 7.3 ± 1.0 (3)                |
| Q451K     | 106 ± 15 (9)          | 11 ± 2 (9)            | 62 ± 3 (4)                     | 5.0 ± 0.4 (5)                |
| R233H/Q451K | 50 ± 8 (6)           | 15 ± 3 (6)            | 15 ± 2 (3)                     | 10 ± 4 (3)                   |
| R233Q/Q451K | 36 ± 5 (6)           | 14 ± 2 (6)            | 22 ± 5 (3)                     | 11 ± 2 (3)                   |
| R233N/Q451K | 39 ± 12 (3)          | 18 ± 8 (3)            | 29 ± 8 (3)                     | 13 ± 2 (3)                   |

**Fig. 3.** cAMP-signaling properties of WT and mutant PPRs. COS-7 cells transfected with the WT (rWT-HA) or a mutant PPR (symbols are shown in the figure key) were treated with the indicated doses of [Tyr34]hPTH-(1–34)NH2 for 30 min at 37°C as described under “Experimental Procedures.” Each curve shows the mean (± S.E.) of data from three to eight experiments, each performed in duplicate. The response of R233N/Q451K mutant (not shown) was identical to that of the R233Q/Q451K mutant.

**Fig. 4.** Inositol phosphate signaling properties of WT and mutant PPRs. Shown are the inositol phosphate levels in transfected COS-7 cells that were untreated (--) or stimulated with 1 μM [Tyr34]hPTH-(1–34)NH2 (--) for 40 min at 37°C. Data (mean ± S.E.) are from one experiment performed in duplicate and representative of three separate experiments. The R233N and R233N/Q451K mutant receptors were inactive in these assays.
these were shifted 10–1000-fold to the right of the WT response (Fig. 3).

Remarkably, cells expressing the double mutant receptors, which bound hPTH-(1–34) with high affinities (Table II), were defective in mediating agonist-induced cAMP production; we detected less than a 2-fold increase in cAMP production in response to a maximum dose (1,000 nM) of hPTH-(1–34) (Fig. 3).

In addition to the adenyl cyclase system, WT PTH/PTHrP receptors also activate phospholipase-C-mediated signal transduction systems (2, 29). Accordingly, PTH-(1–34) (1 μM) stimulated a 4.5-fold increase in inositol phosphate (IP) levels in COS-7 cells expressing rWT-HA, as compared with the basal level of IPs in untreated cells (Fig. 4). No IP response was detected in cells expressing any of the mutant PTH/PTHrP receptors having either individual or combined substitutions in TM 2 and TM 7 (Fig. 4). At high doses (1 μM), the truncated peptide hPTH-(3–34) was inactive in IP assays performed on COS-7 cells expressing either the WT PTH/PTHrP receptor or any of the mutant receptors (data not shown). A small, 7–9-fold increase in cAMP formation was detected for this ligand with rWT-HA and the R233K mutant receptor, but no response was observed with any of the other mutant receptors (data not shown).

DISCUSSION

These studies show that Arg-233 and Gln-451 of the rat PTH/PTHrP receptor play important roles in receptor function. These residues are likely to contribute to interactions involving the amino terminus of the hormone, based on the finding that mutations at these sites markedly impaired the binding of PTH-(1–34) analogs without affecting the binding of PTH-(3–34) analogs. Residues 1–2 of PTH are critical for activating the adenyl cyclase response pathway, as shown by the ability of PTH-(3–34) analogs to antagonize the agonist activity of PTH-(1–34) (30). Although most mutations in the PTH/PTHrP receptor that we have examined so far have had comparable effects on the binding of PTH-(1–34) and PTH-(3–34) ligands, we recently showed that mutations at two sites in the third extracellular loop of the rat PPR (Trp-437 and Gln-440) resulted in similar dissociation of effects on the binding of the respective peptides (27). In other studies, we identified residues in TMs 5 and 6 of the angiotensin II receptor (16) and TM 2 of the substance P receptor (13) that impair binding of agonist ligands but not antagonist ligands. Additional studies will be necessary to directly assess how G protein coupling influences the binding of agonists and antagonists to WT and mutant PTH/PTHrP receptors.

That the two receptor residues that we have identified are highly conserved among the receptors in the PTH/receptor family (Fig. 1) suggests that they perform a function that is preserved in all members of the family. For example, these residues might be involved in maintaining the proper three-dimensional configuration of the membrane-spanning portions of the receptor. Given that the PTH/PTHrP receptor mutants that we have studied here were expressed sufficiently on the cell surface and retained high binding affinity for PTH-(3–34) ligands, it seems unlikely that the mutations severely altered the overall structure of the receptor. Nevertheless, it remains possible that mutations at these sites induce subtle conformational changes that alter other sites more directly involved in ligand binding and signal transduction.

In summary, we have identified two polar residues, Arg-233 in TM 2 and Gln-451 in TM 7 of the rat PTH/PTHrP receptor, that play important roles in determining agonist binding affinity and agonist-induced receptor activation. These residues are functionally linked and may therefore participate in interdomain interactions. Although additional work is needed to fully resolve how these residues contribute to receptor function, our current results demonstrate that receptor mutagenesis strategies combined with the use of agonist and antagonist peptide ligands can help identify and characterize critical residues in these peptide hormone receptors.

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