The N-terminal Region of the Third Intracellular Loop of the Parathyroid Hormone (PTH)/PTH-related Peptide Receptor Is Critical for Coupling to cAMP and Inositol Phosphate/Ca\(^{2+}\) Signal Transduction Pathways*

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Structural determinants within the parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor that mediate G-protein activation of adenylate cyclase and phospholipase C are unknown. We investigated the role of the N-terminal region of the third intracellular loop of the opossum PTH/PTHrP receptor in coupling to two signal transduction pathways. We mutated residues in this region by tandem-alanine scanning and expressed these mutant receptors in COS-7 cells and/or Xenopus oocytes. All mutant receptors retained high affinity PTH binding in COS-7 cells, indistinguishable from wild-type receptors. Receptors with tandem-alanine substitutions in two N-terminal segments (377RLV and 381TKLR) demonstrated impaired adenylate cyclase and phospholipase C activation. Receptor mutants with single-alanine substitutions scanning these two segments showed three different signaling defects in COS-7 cells. 1) Two mutant receptors (V378A and L379A) had reduced inositol phosphate (IP\(_3\)), but normal cAMP responses to PTH. 2) Mutant receptor T381A showed reduced cAMP, but wild-type IP responses to PTH. 3) Mutant receptor K382A demonstrated both markedly reduced cAMP and IP production due to PTH. In oocytes, mutants T381A and K382A showed decreased PTH-stimulated cAMP accumulation and intracellular Ca\(^{2+}\) mobilization. Thus, the N-terminal region of the third intracellular loop of this receptor plays a critical role in coupling to both G\(_{q}\)- and G\(_{q}\)-mediated second-messenger generation.

The parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor is a G-protein-coupled receptor (GPCR) that initiates biological responses to PTH, the major endocrine regulator of calcium and skeletal homeostasis, as well as PTHrP, a cytokine essential for normal endochondral bone formation (1–3). The PTH/PTHrP receptor, by primary sequence alignment, belongs to a distinct subfamily of GPCRs, the PTH/secretin receptor subfamily, which includes receptors for calcitonin, secretin, vasoactive intestinal polypeptide, gastric inhibitory peptide, glucagon, glucagon-like peptide-1 (GLP-1), corticotropin-releasing factor, pituitary adenylate cyclase-activating polypeptide (PACAP), and the insulin diuretic hormone (4). The PTH/PTHrP receptor mediates its actions by coupling to second messenger generation (cAMP, inositol trisphosphate (IP\(_3\)), and intracellular Ca\(^{2+}\)), presumably through activation of G\(_{q}\) and G\(_{q}\)-like proteins (5, 6). The ability to generate second messengers through two signaling pathways is a distinct functional property shared among many members of the PTH/secretin receptor subfamily. Thus far, the structural features responsible for coupling to dual signaling pathways are unknown for any receptor in this subfamily.

Structure-function studies of signal transduction properties of GPCRs have been best described for members of the rhodopsin/adrenergic receptor subfamily. Overall, these studies have shown that all intracellular regions of the receptors contribute to the domains which couple to signaling pathways (7, 8). Four intracellular regions (the N- and C-terminal portions of the third intracellular (IC-3) loop, the C-terminal portion of the IC-2 loop, and N-terminal portion of the cytoplasmic tail) have all been shown to play a critical role in G-protein coupling (7, 8). Direct sequence comparisons of these intracellular regions between members of the PTH/secretin and the rhodopsin/adrenergic receptor subfamilies, however, have revealed no sequence homologies in these regions. This, therefore, excludes the possibility of identifying shared sequence motifs required for G-protein coupling among these receptors.

Studies of signal transduction mediated by the PTH/secretin receptor subfamily have, thus far, provided limited information. Studies comparing different splice variants of members of this subfamily suggested that inserted sequences in the N-terminal region of the IC-1 loop and the C-terminal region of the IC-3 loop could play a role in modulating receptor-G-protein coupling. Insertions in the IC-1 domain of receptors for both calcitonin and CRF reduced agonist-stimulated responses (9, 10). In these instances, insertions at these positions seem selectively to disrupt normal G-protein coupling. Similarly, insertions in the C-terminal region of the IC-3 loop of the PACAP receptors cause differential activation of either cAMP or IP\(_3\) production (11). Insertions in this region also modulate cAMP
and IP₃ signaling in a negative manner. Since most of the receptors in the PTH/secretin subfamily do not have insertions at this position and can activate both signaling pathways, it is likely that the major structural determinants responsible for coupling the receptor to these pathways are not present in the insertion sequences, but are located elsewhere in the receptor.

Recent studies have examined the role of the cytoplasmic tail and the IC-1 loop of the PTH/PTHrP receptor in signal transduction. Using mutant receptors bearing truncation and tandem-alanine substitutions, we have shown that the cytoplasmic tail of the PTH/PTHrP receptor is not required for binding and adenylate cyclase or phospholipase C activation. Most of the cytoplasmic tail can be deleted without loss of these functions (12). Other groups have suggested that the cytoplasmic tail of the receptor could play a regulatory role in limiting indiscriminatory coupling to G-proteins (13, 14). Recently, a tail of the PTH/PTHrP receptor is not required for binding and the IC-3 loop of the receptor in coupling to both adenylate cyclase and phospholipase C activation. The importance of this residue in signaling was also illustrated by our studies (16). Different mutations of an equivalent site in opossum kidney PTH/ PTHrP receptor resulted in defective receptor expression (H220A) and signal transduction (H220D).

Site-directed mutagenesis studies involving the IC-3 loop of the GLP-1 receptor have recently been reported (17). The N-terminal domain of the IC-3 loop of this receptor (specifically residue Lys⁸³⁴) is required for efficient coupling of the GLP-1 receptor to stimulation of the adenylate cyclase activity. It is unclear, however, whether Lys⁸³⁴ is also involved in coupling to phospholipase C activation. In the present study, we systematically investigated the role of the N-terminal region of the IC-3 loop of the PTH/PTHrP receptor in coupling to both adenylate cyclase and phospholipase C activation. We introduced tandem- and single-alanine mutations in the N-terminal region of the IC-3 loop by PCR-mediated mutagenesis. Mutant receptors were transiently expressed in COS-7 cells and assessed for PTH binding, CAMP accumulation, and IP production. In addition, cRNAs encoding these mutant receptors were microinjected into Xenopus oocytes, and CAMP accumulation and intracellular Ca²⁺ mobilization were assessed. These studies demonstrate that the N-terminal region of the IC-3 loop of the PTH/PTHrP receptor is critical for coupling to G-protein-mediated signal transduction pathways and that specific hydrophobic, hydrophilic, and positively charged residues in this region of the IC-3 loop play a crucial role in coupling to adenylate cyclase and phospholipase C activation.

EXPERIMENTAL PROCEDURES

**Materials—** Synthetic bPTH(1–34) and hPTHrP(1–34) were purchased from Bachem, Inc. (Torrance, CA). Restriction enzymes and T4 DNA ligase were purchased from Life Technologies, Inc and Promega (Madison, WI). Sequencing 2.0 sequencing kit and myosin-6 (myosin-Iso) (TRK912, 5 mCi/ml) were obtained from Amersham Life Science, Inc. (Cleveland, OH). Cell culture media and reagents were obtained from the UCSF Cell Culture Facility. Oligonucleotides were synthesized and purified at UCSF Biomolecular Resource Facility. Adult female Xenopus laevis were purchased from Xenopus I (Ann Arbor, MI). The M-CAP kit, T7 RNA polymerase, the cap analog (5’-7-methyl GpppG) and Phe DNA polymerase were from Stratagene (La Jolla, CA). 45CaCl₂ (NE-Z, 9 mCi/ml) was from DuPont NEN. DEAE-dextran, chloroquine, and collagenase type IA were from Sigma. QIAEX II and QIAGEN columns were purchased from QIAGEN, Inc. (Chatsworth, CA).

**Mutagenesis by Overlapping Extension PCR—** Tandem-alanine mutant PTH/PTHrP receptor constructs were generated by overlapping extension PCR, as described by Pease and co-workers (18). To introduce each tandem-alanine mutation, four PCR primers were used. The same two external primers were used to construct all of the mutants. The upstream external primer contained about 20 nucleotides of the coding sequence located about 50 nucleotides upstream of the restriction site for NsiI in the coding region of the PTH/PTHrP receptor. The downstream external primer was derived from the opposite DNA strand and located about 50 nucleotides downstream of the restriction site for BstNI in the polylinker region of the pcDNA1/Amp vector. Two internal primers were based on the opposite DNA strands and contained complementary sequences of 12 nucleotides at the 5’ end encoding four tandem-alanine residues. The 3’ region of the upstream internal primers contained about 20 nucleotides of the noncoding strand sequence upstream of the restriction site. The 3’ region of the downstream internal primers contained 20 nucleotides of the coding strand sequence downstream of the mutation sequence. Primers were designed with melting temperatures between 55 and 65 °C.

Overlapping-extension PCR was carried out in two steps. First, two PCRs were performed using the opossum kidney PTH/PTHrP receptor cdNA in pcDNA1/Amp (OK02) as a template (original plasmid kindly provided by Dr. Harold Jumper, Harvard Medical School). In the first PCR, the upstream external and an upstream internal primers were used to generate the upstream fragment. In the second PCR, the downstream external and a downstream internal primers were used to generate the downstream fragments. The resulting PCR products were separated by agarose gel electrophoresis and extracted with QIAEX II. Second, PCR was performed using the two external primers and a fraction of the purified upstream and downstream fragments. At this stage, but significant frequency, two overlapping strands of the upstream and downstream fragments were annealed and extended by the DNA polymerase to generate a full-length template, which was then amplified by the two external primers. PCR products were separated by agarose gel electrophoresis and extracted by QIAEX II, digested with BstNI and NsiI and ligated into the SpH1-NsiI sites of the predigested OKOII vector. Recombinant vectors were transformed into Escherichia coli strain Top10F′ (Invitrogen, San Diego, CA) and purified using QIAGEN columns according to the manufacturer’s recommended procedure. The presence of the desired mutations and the fidelity of surrounding sequences generated by PCR were confirmed by DNA sequencing.

**Cell Culture and Transient Transfection by DEAE-dextran—** COS-7 cells were maintained in tissue culture flasks (75 cm²) containing Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum. COS-7 cells were transfected by the DEAE-dextran method (19). Briefly, the cells were split at a 1:5 ratio the day before transfection. For each flask, DNA (5 µg) was mixed with DEAE-dextran, chloroquine, and DMEM supplemented with 2% Nu-serum, and the mixture was incubated with cells for 3 h at 37°C. The cells were then treated with 10% Me₂SO for exactly 2 min, followed by washing with phosphate-buffered saline (pH 7.5). Cells were incubated for 24 h, then split into six wells (30 mm in diameter) and cultured for an additional 48 h before PTH treatment.

**Radioigid Binding Assay—** Radioiodinated ligand binding assays were carried out as described previously (20). Briefly, 72 h after transfection, COS-7 cells were preincubated in DHB (DMEM containing 20 mM HEPES (pH 7.4) and 0.1% bovine serum albumin) at 37°C for 1 h. Cells were then incubated with 1 mCi of DHB containing 50,000 cpm of 125I-labeled 1-34 PTHR(1-34), plus varying concentrations of the appropriate unlabeled peptides for a 1-h incubation at room temperature. The cells were then washed and collected in 1.5 ml of 0.8 N NaOH. Bound 125I-labeled PTHrP was quantified in a γ-counter. Specific binding was expressed as percent radioligand bound over total radioligand added. Results are expressed as the mean ± S.E. with n representing the number of individual experiments. Scatchard analysis were performed under similar conditions as described previously (16). Briefly, COS-7 cells were incubated with ligand at 4 °C for 2 h in 12-well plates containing 3 µCi/PTHrP(1-34) (50,000 cpm/well) and different concentrations of unlabeled hormone. Scatchard analysis of binding data was performed using the Ligand program (G. A. McPherson, Elsevier Science Publishers, Amsterdam).

**Studies of cAMP Production in COS-7 Cells—** Experiments assessing cAMP accumulation and the CAMP RIA were performed as described (20). Briefly, COS-7 cells were incubated with 10 mCi in medium containing 0.4 mM isobutylmethylxanthine, followed by 10 min in DHB containing appropriate concentrations of PTH. Cells were washed with cold phosphate-buffered saline, and cAMP was extracted with 95% ethanol and assayed by RIA. Data are expressed as the mean ± S.E. with n representing the number of individual experiments. Results are expressed as the mean ± S.E. with n representing the number of individual experiments. Scatchard analysis were performed under similar conditions as described previously (16). Briefly, COS-7 cells were incubated with ligand at 4 °C for 2 h in 12-well plates containing 10 µCi/PTHrP(1-34) (50,000 cpm/well) and different concentrations of unlabeled hormone. Scatchard analysis of binding data was performed using the Ligand program (G. A. McPherson, Elsevier Science Publishers, Amsterdam).
COS-7 cells transiently expressing wild-type (WT) and mutant PTH/PTHrP receptor constructs were performed as follows. Briefly, 48 h after transfection, COS-7 cells were labeled with myo-[3H]inositol (2 μCi/ml) in DMEM supplemented with 10% fetal bovine serum for 18–24 h (21). Cells were washed with DHB and preincubated with 5 mM LiCl in DHB for 10 min at 37 °C. Cells were then stimulated with different concentrations of bPTH-(1–34) in DHB containing 5 mM LiCl for 45 min at 37 °C. Total IPs were extracted from cells with an extraction solution of 20 mM LiCl at 1.5 ml/well for 1 h at 4 °C. After neutralization of the extracts to pH 7.5 with NH₄OH, they were loaded on Dowex AG 1-X8 columns. Total IPs (IP₁, IP₂, IP₃, and IP₄) were eluted with a solution of 2 M ammonium formate and 20 mM formic acid. Previous studies using [³H]IP₁, 1,4-IP₂, 1,4,5-IP₃, and 1,3,4,5-IP₄ competed for by bPTH-(1–34) (0–10 of independent experiments performed. Values with asterisks are significantly different from WT (CaCl₂, 0.41; and HEPES, 10 (pH 7.6).

Preparation of Receptor cRNAs and Injection into Xenopus Oocytes—Briefly, WT and mutant PTH/PTHrP receptor constructs were subcloned into the SmaI-XhoI sites of the pGEM-HE vector (22) kindly provided by Dr. Emily Liman (Harvard Medical School) as detailed previously (12). After linearization with NheI, cDNA templates were in vitro transcribed with T7 RNA polymerase using the mCAP kit. cRNAs were ethanol-precipitated, washed, and resuspended in diethylpyrocarbonate-treated water prior to agarose gel electrophoresis and quantification by A₂₆₀ readings.

Anesthesia, surgery, and injection of oocytes from female Xenopus laevis were performed as described previously (12) and approved by the San Francisco Department of Veterans Affairs Medical Center Animal Welfare Subcommittee. All experiments were conducted using a modified Barth’s saline solution buffered with HEPES (MBSH) containing (in mM): NaCl, 88; KCl, 1; NaHCO₃, 2.4; MgSO₄, 0.82; Ca(NO₃)₂, 0.3; CaCl₂, 0.41; and HEPES, 10 (pH 7.6).

Fig. 1. Schematic diagram of four mutant PTH/PTHrP receptors bearing tandem-alanine mutations in the N-terminal region of the IC₃ loop. The N-terminal region of the IC₃ loop of the PTH/PTHrP receptor was systematically scanned by tandem-alanine substitutions by overlapping PCR. Four tandem-alanine mutants (C0, C1, C2, and C3) were generated, in which 4 consecutive residues in the N-terminal region of the IC₃ loop were replaced by alanine.

Table I

| Sequence | Maximal binding IC₅₀ (nm) | Maximal cAMP (pmol/well) EC₅₀ (nm) |
|----------|--------------------------|-----------------------------------|
| WT       | 22.2 ± 3.4 (9)           | 3.6 ± 0.5                        | 105.7 ± 11.3 (19) | 0.15 ± 0.03 |
| C0       | 21.6 ± 4.9 (4)           | 5.0 ± 1.7                        | 68.2 ± 11.1* (10) | 0.69 ± 0.17* |
| C1       | 21.9 ± 2.1 (6)           | 2.1 ± 0.4                        | 43.7 ± 6.3* (10)  | 0.66 ± 0.15* |
| C2       | 20.9 ± 2.5 (3)           | 3.5 ± 1.0                        | 92.7 ± 18.0 (8)   | 0.18 ± 0.05 |
| C3       | 19.3 ± 3.2 (3)           | 3.4 ± 0.8                        | 110.9 ± 16.2 (6)  | 0.07 ± 0.02 |

PIT Binding, cAMP Accumulation, and IP Production in COS-7 Cells—To assess the role of the N-terminal region of the IC₃-3 loop in signaling, we used a strategy called tandem-alanine scanning mutagenesis, in which 4 consecutive amino acid residues are replaced by alanines (Fig. 1). Alanine is chosen because it lacks an amino acid side chain beyond the β carbon. If critical receptor-G-protein interactions are through side chains in the loop, then such contacts are eliminated by alanine substitution, and such mutations will likely result in impaired light efficiency as described (12). Oocytes were maintained in MBSH in sterile Petri dishes at 19 °C for 48 h after injection and prior to assessing [⁴Ca] efflux or cAMP accumulation. Incubation media were replaced with fresh MBSH after 24 h. Any visibly damaged or motile oocytes were discarded at 24 and 48 h after injection.

[⁴Ca] Efflux Assay in Oocytes—[⁴Ca] efflux from prefilled Xenopus oocytes has been used to assess intracellular Ca²⁺ mobilization by several GPCRs expressed in this system (23–26). Receptor cRNA- or water-injected control oocytes were labeled with [⁴Ca]Cl₂ and exposed to bPTH-(1–34) as detailed previously (12). [⁴Ca] efflux assays were conducted in triplicate in cluster/24-well culture plates. Basal [⁴Ca] efflux from 5 oocytes was determined by collecting the media surrounding the oocytes and averaging the isotope released in three consecutive 20-min collection periods. Thereafter, MBSH containing bPTH-(1–34) (5 μg/ml) was added to the oocytes. The media were collected and replaced with fresh MBSH plus bPTH-(1–34) for a total of three 20-min treatment periods. All experiments included oocytes injected with WT PTH/PTHrP receptor cRNA as an internal control. Results for both basal and PTH-stimulated [⁴Ca] efflux are expressed as mean ± S.E. Basal [⁴Ca] efflux typically ranged between 100 and 500 cpm/oocytes/20-min interval. Responses to PTH are expressed as either the net [⁴Ca] efflux per 60 min (treated – basal) or as the -fold increase in [⁴Ca] efflux (net [⁴Ca] effluxed in 60 min/basal [⁴Ca] efflux in 20 min).

cAMP Accumulation Assay in Oocytes—cAMP accumulation in cRNA-injected or noninjected oocytes was assessed in triplicate using a modification of published procedures (27, 28). Briefly, groups of 13–15 oocytes were washed three times with MBSH and aliquoted into wells of a cluster/24-well plate. Fresh MBSH containing isobutylmethylxanthine (4 × 10⁻⁴ M) (1.0 ml) with or without bPTH-(1–34) (3 μg/ml) was added to the different groups of oocytes. After 30 min at room temperature, the media were aspirated, and ice-cold absolute ethanol (1.0 ml) was added. Eggs were gently swirled in the ethanol, which was then removed to 12 75-mm glass tubes. This extraction procedure was repeated with two additional 1-ml aliquots of ethanol, and all ethanol extracts were pooled and air-dried overnight. cAMP in the residue was resuspended in 0.3 ml of sodium acetate (0.05 M, pH 6.2) and measured by radioimmunoassay (12). Results are expressed as the average -fold increase in cAMP accumulation in response to PTH/basal cAMP content in unstimulated oocytes.

Our extraction and radioimmunoassay were validated for cAMP measurements in oocytes as follows. 1) The extraction procedure quantitatively removed the majority of CAMP present, since groups of oocytes crushed and re-extracted with ethanol by this method did not contain cAMP that was measurable by our radioimmunoassay (detection limit: 0.05 pmol/0.1 ml). 2) CAMP in extracts of oocytes diluted in parallel with the cAMP standard curve (data not shown). 3) By spiking cAMP extracts with known quantities of CAMP, we found that any impurities in these extracts did not interfere with the accurate detection of CAMP in the radioimmunoassay (data not shown).

RESULTS

PTH Binding, cAMP Accumulation, and IP Production in COS-7 Cells—To assess the role of the N-terminal region of the IC₃-3 loop in signaling, we used a strategy called tandem-alanine scanning mutagenesis, in which 4 consecutive amino acid residues are replaced by alanines (Fig. 1). Alanine is chosen because it lacks an amino acid side chain beyond the β carbon. If critical receptor-G-protein interactions are through side chains in the loop, then such contacts are eliminated by alanine substitution, and such mutations will likely result in impaired CAMP production in COS-7 cells expressing WT and tandem-alanine mutants of PTH/PTHrP receptors.

COS-7 cells transfected with WT or mutant receptor cDNA were assayed for PTH binding and PTH-stimulated cAMP production 72 hrs after transfection as described under "Experimental Procedures." [³H]bPTH-P (1–34) (50,000 cpm/well) was used as the radioligand, which was competed for by bPTH-(1–34) (0–10⁻⁶ M). Transfected COS-7 cells were treated with bPTH-(1–34) (0–10⁻⁶ M) in the presence of isobutylmethylxanthine (0.4 mM) for 10 min at room temperature, and cAMP was extracted and measured by RIA. Numbers in parentheses refer to the number of independent experiments performed. Values with asterisks are significantly different from WT (p < 0.05).
receptor signaling. Another reason for choosing alanines is that this substitution avoids introducing steric hindrance or unwanted ionic interactions. Both are likely to occur when amino acid residues with bulky side chains or charged groups are introduced.

Four tandem-alanine mutant receptors were generated by PCR-based site-directed mutagenesis. Radiolabeled 125I-PTHrP binding and PTH-stimulated cAMP and total IP production were determined in transiently transfected COS-7 cells. All four mutants were well expressed and showed similar levels of specific 125I-labeled PTHrP-(1-34) binding, which was approximately 20% of added 125I-PTHrP-(1-34) and indistinguishable from that of the WT receptor (Table I). Apparent binding affinities in these mutants, as assessed by half-maximal displacement, were also similar to that of the WT receptor (IC50 (nM) = 3.6 ± 0.5). Mutations in the two N-terminal segments C0 (377RVLA380) and C1 (381TKLR384), however, caused significant reduction in maximal PTH-stimulated cAMP production, which were 68% and 37% of that of the WT receptor, respectively (Fig. 2). These same mutations raised the IC50 values by 4–5-fold over that of WT receptors (EC50 (nM) = 0.15 ± 0.03). The remaining two mutants (C2 and C3) displayed maximal cAMP responses and EC50 values similar to those of WT PTHrP receptors (Table I).

The impaired cAMP responses of C0 and C1 suggested that the N-terminal region of the receptor might play a critical role in Gs-mediated signaling. To investigate whether this same region might also play a role in Gq-mediated signaling, the ability of these mutants to initiate PTH-stimulated IP production was assessed. Both mutant C0 and C1 showed marked reductions in PTH-stimulated IP production (to about 15% of that of WT receptor) (Fig. 3). The other mutants tested (C2 and C3) retained the ability to activate phospholipase C equivalently to 50% or greater of WT responses (data not shown).

To pinpoint individual residues in C0 and C1 segments critical for signaling, seven single-alanine mutants scanning C0 and C1 were generated (Figs. 4A and 5A), and their signaling properties were examined. As expected, mutating these 7 residues to alanine singly did not affect PTH binding, either in terms of maximal binding capacity (Figs. 4B and 5B) or binding affinity (Tables II and III). Surprisingly, unlike the tandem-alanine mutant C0, all three single-alanine mutants (C0–1, C0–2, and C0–3) showed WT cAMP responses, in terms of both maximal cAMP levels and half-maximal responses to PTH (Fig. 4 and Table II). This indicates that these sites in C0, individually, are not critical in Gs coupling to adenylate cyclase activation but collectively they are important in coupling to this pathway. Two of these mutants, C0–2 (V378A) and C0–3 (L379A), however, did show markedly reduced PTH-stimulated IP production, which was 29% and 19% of that of the WT receptor, respectively (Fig. 4). Thus, the ability of these mutant receptors to couple Gs, or a similar G-protein to phospholipase C activation is severely impaired.
In contrast to the single-site mutants of C0, introduction of single-alanine mutations into the C1 segment of the receptor (C1–1, C1–2, C1–3, and C1–4) altered both adenylate cyclase and phospholipase C activation. Two of the mutants, C1–1 (T381A) and C1–2 (K382A), showed reduced maximal levels of cAMP production, which were 60% and 10% of that of WT receptor, respectively (Fig. 5). The other two mutants, C1–3 (L383A), and C1–4 (R384A), showed WT maximal cAMP responses (Fig. 5). In contrast to results with the tandem-alanine mutant C1 (TKLR), whose EC50 value for adenylate cyclase activation was approximately 4-fold higher than that of the WT receptor (Fig. 5). The other three mutants (C1–1, C1–3, and C1–4) were equivalent to the WT receptor in initiating PTH-stimulated phospholipase C activation.

Scatchard Analysis of WT and Mutant PTH/PTHrP Receptors—Since the magnitude of signaling responses of GPCRs can be influenced by levels of receptor expression (21), we assessed the expression of WT and mutant PTH/PTHrP receptors in our transfected cells to determine whether the altered signaling properties observed with mutant receptors could be due to differences in receptor expression. To assess cell-surface receptor content, we carried out PTH binding assays at 4°C and quantitated mutant receptor expression by Scatchard analysis. As shown in Table IV, mutant receptors of interest, whether they showed WT signaling (C0–1) or impaired signaling (C0–2, C0–3, and C1–2), were expressed at levels similar to those of WT PTH/PTHrP receptors in our transient transfection system: ~106 receptors/cell. This indicates that the defects in signaling we observed were not due to decreased receptor expression, but probably to an intrinsic signaling defect due to the mutation.

Signal Transduction of WT and Mutant PTH/PTHrP Receptors in Xenopus Oocytes—Our previous studies demonstrated that PTH/PTHrP receptors can be expressed in oocytes and couple to intracellular Ca2+ mobilization, as reflected by increases in 45Ca efflux (12). A distinct advantage of oocytes is that Ca2+ mobilization responses are dramatic (i.e. 10–20-fold increases), compared to the typical ~1.7-fold increases in [3H]IP production in transiently transfected COS-7 cells. We, therefore, tested the ability of these PTH/PTHrP receptor mutants to stimulate intracellular Ca2+ mobilization and cAMP accumulation in this system.

As shown in Fig. 6, there was an ~13–14-fold increase in 45Ca efflux in response to bPTH(1–34) in oocytes injected with WT PTH/PTHrP receptor cRNA. In contrast to results in COS-7 cells, oocytes expressing only C1–1 and C1–2 receptor cRNAs demonstrated reduced ability to stimulate intracellular Ca2+ mobilization after addition of PTH (Fig. 6). 45Ca efflux rose by only ~3- and ~7-fold in oocytes expressing C1–1 and C1–2 receptor cRNA, respectively. This is significantly lower than WT 45Ca efflux responses (p < 0.001 and p < 0.05, respectively). Eggs injected with the mutant C0–2 receptor cRNA demonstrated an ~8-fold increase in 45Ca efflux, which was lower than the 13–14-fold increase seen in oocytes expressing WT PTH/PTHrP receptors.
WT receptors. This difference, however, did not reach statistical significance (Fig. 6). Thus, two residues (Thr381 and Lys382) in this portion of the IC-3 loop of the PTH/PTHrP receptor play particularly important roles in mediating efficient PTH-induced Ca\(^{2+}\) mobilization in oocytes.

The above studies indicate that there are differences in which residues of the receptor are critical for promoting G-protein interactions with phospholipase C in oocytes compared to COS-7 cells. To determine whether the same difference was observed with the activation of G\(_\alpha\), we examined PTH-stimulated cAMP production in oocytes. cAMP rose by 7.8-fold (n = 5) and 9.8 ± 3.2-fold (n = 3) in oocytes expressing WT PTH/PTHrP receptor cRNA in the experiments involving C0 and C1 point mutations, respectively. Mean basal cAMP content was 143 ± 23 and 202 ± 18 fmol/oocyte for experiments with C0 and C1 mutants, respectively. Results significantly reduced compared to WT responses are shown as * (p < 0.05). Noninjected control oocytes demonstrated no cAMP accumulation above basal levels after the addition of bPTH(1–34) (data not shown).

**DISCUSSION**

A key issue in understanding the physiologic functions of a receptor is to determine the structural basis for its activation of intracellular signal transduction. In the case of the rhodopsin/\(\beta\)-adrenergic receptor subfamily, the IC-3 loop has been identified as an important structural domain, whose residues couple to G-proteins and determine specificity of receptor-G-protein interaction (7, 8). Studies using chimeric receptors from this subfamily have shown that the specificity of receptor-G-protein coupling can be altered by swapping portions of the IC-3 loops between these receptors. Coupling of receptors to G\(_\alpha\)-adenylate cyclase activation can be switched to G\(_\beta\)-phospholipase C signaling pathway (29, 30). In the PTH/secretin receptor subfamily, the role of the IC-3 loop in signal transduction has not been defined. Many receptors in this subfamily at physiologic levels of expression in their target cells can couple to two signaling pathways (1, 4, 5). This study examines the role of the IC-3 loop in coupling the PTH/PTHrP receptor to G-proteins and attempts to identify residues that are important in differentially activating the two signal transduction pathways.

In these experiments, the N-terminal region of the IC-3 loop of the PTH/PTHrP receptor was scanned by site-directed mutagenesis. The effect of mutating the key residues on PTH binding, cAMP accumulation, and IP production was assessed. We demonstrated that this region of the receptor is critically important in coupling the PTH/PTHrP receptor to both G\(_\alpha\)-adenylate cyclase and G\(_\beta\)-phospholipase C signaling pathways. In COS-7 cells transiently transfected with receptor mutants, it was the hydrophobic residues Val378 and Leu379 that proved critical for phospholipase C activation, while the hydrophilic residue Thr381 was important for adenylyl cyclase stimulation and the positively charged residue Lys382 was key for activation of both effector pathways. In contrast, in Xenopus oocytes, the latter two sites, Thr381 and Lys382, proved to be pivotal for activation of both cAMP accumulation and intracellular Ca\(^{2+}\) mobilization. Thus, there appear to be differences in the specific residues involved in coupling to G-proteins, which activate

**TABLE IV**

Scatchard analysis of WT and single-alanine mutant PTH/PTHrP receptor cRNAs expressed in transiently transfected COS-7 cells

| Sequence       | Receptor no/cell | Kd (nM) |
|----------------|------------------|---------|
| WT             | RVLATK           | 0.86 ± 0.01 | 2.3 ± 0.1 |
| C0–1           | Arg → Ala        | 0.90 ± 0.03 | 4.1 ± 0.2 |
| C0–2           | Val → Ala        | 0.88 ± 0.13 | 2.9 ± 0.2 |
| C0–3           | Leu → Ala        | 0.80 ± 0.00 | 2.4 ± 0.1 |
| C1–2           | Lys → Ala        | 1.05 ± 0.25 | 2.8 ± 1.3 |

**Fig. 6.** \(^{45}\)Ca efflux responses to the addition of bPTH(1–34) in oocytes expressing WT and mutant PTH/PTHrP receptor cRNAs. Xenopus oocytes were injected with either WT or mutant PTH/PTHrP cRNA (50 ng/egg), and \(^{45}\)Ca efflux was determined as described under “Experimental Procedures.” Mean basal \(^{45}\)Ca efflux is shown as cells/20 min and averaged 329 ± 12 and 223 ± 22 cpm for the C0 (panel A) and C1 point mutants (panel B), respectively. The stimulation of \(^{45}\)Ca efflux in response to 60 min of incubation with bPTH(1–34) (3 \(\mu\)g/ml) is shown for each tandem-alanine mutant of C0 and C1 in panels A and B, respectively. Data are presented as net PTH-stimulated \(^{45}\)Ca efflux (cpm/60 min) as detailed under “Experimental Procedures.” The results shown represent the averages of four to six independent experiments performed in triplicate on separate batches of oocytes from different animals. Responses to PTH were significantly lower in the mutants shown, compared to oocytes expressing WT PTH/PTHrP receptor cRNA (n = 6 or 8) as depicted by * (p < 0.05) and ** (p < 0.001).

**Fig. 7.** cAMP accumulation in oocytes expressing WT and mutant PTH/PTHrP receptor cRNAs. Oocytes were injected with different cRNAs (~ 50 ng/40 nl), and cAMP production was measured after extraction of oocytes and assayed by RIA as described under “Experimental Procedures.” Results are shown as the average fold increase in cAMP content in response to bPTH(1–34) compared to the basal cAMP content in eggs not treated with hormone in three or five (panel A) or three independent experiments (panel B). PTH stimulated cAMP production by 7.8 ± 1.5-fold (n = 5) and 9.8 ± 3.2-fold (n = 3) in oocytes expressing WT PTH/PTHrP receptor cRNA in the experiments involving C0 and C1 point mutations, respectively. Mean basal cAMP content was 143 ± 23 and 202 ± 18 fmol/oocyte for experiments with C0 and C1 mutants, respectively. Results significantly reduced compared to WT responses are shown as * (p < 0.05). Noninjected control oocytes demonstrated no cAMP accumulation above basal levels after the addition of bPTH(1–34) (data not shown).
the same effector pathways in these two commonly employed expression systems. This finding may be due to differences in the activation domains within the α-subunits of G-proteins, the G-protein subunits responsible for activation of the effectors (e.g. βγ-subunits versus α-subunit), or effector enzymes themselves in the two cell systems (31). In support of the former, studies have shown that the Gα subunit in Xenopus differs substantially from mammalian Gα subunits, suggesting that primary sequence differences between these and other α-subunits could underlie the signaling differences we observed (32).

The overall results of our study with the PTH/PTHrP receptor agree with results obtained with the GLP-1 receptor in that the N-terminal region of the IC-3 loop of the latter receptor is critical for coupling to cAMP production. Furthermore, the lysine residue at position 382 in the PTH/PTHrP receptor, which is equivalent to residue Lys382 in the GLP-1 receptor, plays a key role in coupling to this signaling pathway (17). Our studies, which included more residues in the N-terminal region of the PTH/PTHrP receptor than those scanned in the GLP-1 receptor, also identified a threonine residue (Thr381), which is important in coupling the PTH/PTHrP receptor to adenylyl cyclase activation. It is not known whether there is a residue with an equivalent function in the GLP-1 receptor.

Regarding phospholipase C activation, there appear to be significant differences between PTH/PTHrP and GLP-1 receptors. Our results indicate that the N-terminal region of IC3 loop of the PTH/PTHrP receptor is also critical for coupling to IP3/Ca2+ signaling pathways with residue Lys382 being central to phospholipase C activation in both expression systems. Block deletion of residues in the N-terminal region of the IC-3 loop of the GLP-1 receptor did not alter GLP-1-stimulated Ca2+ mobilization in stably transfected CHO cells (17). Whether the differences between key residues involved in signal transduction for PTH/PTHrP and GLP-1 receptors are due to inherent sequence differences between the two receptors, technical differences due to the strategies employed (alanine scanning versus deletion mutation), or the variable sensitivity of the assays used (IP production versus intracellular Ca2+ measurements) is unclear. It would be of great interest to determine whether a K334A mutant of GLP-1 receptor, a position that is highly conserved within the PTH/secretin receptor subfamily, would affect the ability of the GLP-1 receptor to activate phospholipase C.

Two patterns of impaired Gα-mediated cAMP responsiveness were observed in experiments with COS-7 cells transfected with mutant PTH/PTHrP receptor constructs. 1) The maximal response to PTH was decreased (e.g. C1–2). 2) The maximal cAMP response to PTH was reduced, and there was a modest shift to the right in the dose-response curve (EC50) for PTH (e.g. C1). One interpretation for these results is that the maximal response reflects the maximal number of PTH/PTHrP receptors that are coupled to Gα and can be activated by PTH. This parameter serves as an index of functional Gα-receptor complex formation, while the EC50 value indicates activation efficiency. The EC50 quantitates the ability of the receptor to promote Gα coupling to downstream effectors. According to this interpretation, the observation that C1–2 exhibits a 90% decrease in maximal response, with little change in EC50, suggests that there are only a limited number of mutant receptors that are associated with Gα. Once functional Gα-receptor complexes are formed, however, mutant receptors act like WT receptors in their ability to activate Gα. The role of this particular residue, therefore, is consistent with an association with but not a direct activation of Gα.

In contrast to C1–2, the mutant C1 showed an approximately 4-fold increase in its EC50 value. This suggests that the ability of this mutant receptor to activate Gα is decreased by 4-fold. Therefore, to achieve a level of activation of Gα comparable to that induced by WT receptors, higher doses of PTH are needed. Increased ligand is required to transform more receptors into a state capable of activating Gα to compensate for the lower activation efficiency of this mutant. The fact that the cAMP response of this mutant plateaued at 40% of the WT level, with only a small increase (4-fold) in the EC50 value, further suggests that the number of functional Gα-receptor complexes formed may also be limited. Thus, this type of mutation has two effects: 1) to decrease the ability of the receptor to activate Gα, and 2) to reduce the functional Gα-receptor complex formation.

The two different functional effects of the mutant receptors appear to correlate with the types of mutations introduced. In the C1 tandem-alanine mutant, all the side chains of 4 consecutive residues were eliminated. These mutations may alter the interaction not only between the receptor and G-proteins but also between regions of the receptor itself. A receptor with 4 consecutive alanines may be more flexible than the WT receptor and able to assume multiple local conformations, permitting its association with G-proteins. This conformational flexibility may, however, affect activation efficiency, as evidenced by an increase in the EC50 values. Such a tandem-alanine mutant could also have a less productive association with G-proteins, which would be demonstrated in reduced maximal signal transduction responses (e.g. C0 and C1). These effects could be due to the loss of key residues themselves (C1) or of the adjacent residues (C0). In contrast, single-alanine mutations eliminate only 1 residue in the receptor, thus maintaining the interaction of neighboring residues with other portions of the receptor. As in the case of mutant C1–2, the substitution of alanine at residue Lys382 resulted in the loss of a positively charged side chain, which is critical for association with Gα or Gβγ. The side chains of other residues in the receptor, which are involved in interaction with Gα, however, are not changed. Therefore, once Gα is bound to the receptor, Gα activation proceeds normally as evidenced by the EC50 for this mutant, which is indistinguishable from WT. The increased flexibility, as a result of substitution of 4 alanine residues, is more consistent with the functional phenotypes we observed with tandem-alanine mutants C0 and C1 than with those of single-alanine mutants C1–1 and C1–2.

One of the hallmarks of the PTH/secretin receptor subfamily is that many receptors in this subfamily activate dual signal transduction pathways (4, 5). Our studies have shown that the N-terminal region of the IC-3 loop of the PTH/PTHrP receptor is critical for differential stimulation of both cAMP and IP production. Mutations of specific residues in this portion of the receptor altered its ability to activate one signaling pathway over the other in COS-7 cells. Elimination of hydrophobic side chains, by mutation of valine and leucine to alanine (V378A and L379A), impaired the ability of the receptor to activate phospholipase C. Such mutations, however, did not affect ability of the receptor to activate adenylyl cyclase. In contrast, mutation of the hydrophilic threonine residue to alanine (T381A) showed an opposite effect: impaired cAMP production but no effect on IP generation in COS-7 cells. Similarly, in oocytes, the point mutant V378A demonstrated impaired coupling to Ca2+ mobilization, but normal stimulation of cAMP production. These results overall demonstrate that the same effector can cause differential effects on Gα-adenylyl cyclase and Gα-phospholipase C signal pathways. The simplest explanation for this differential signaling may be that different sets of residues within the receptor are involved in coupling to Gα and Gβγ or Gβγ-like proteins.

If this is true, then how does one receptor couple to both PTH and GLP-1? Evidently, only a small subset of residues is critical for coupling to both Gα and Gβγ. The results of our study suggest that the interaction of these residues with Gα and Gβγ may be dependent upon their relative spatial orientation in the receptor. This may explain why a single alanine substitution results in decreased Gα coupling but increased Gβγ coupling. Future studies will be necessary to determine the specific residues that are involved in coupling to Gα and Gβγ, and how their spatial arrangement in the receptor affects Gα versus Gβγ coupling.

Given the importance of the N-terminal region of the IC-3 loop in coupling to Gα, it is possible that other receptors in this subfamily may also utilize this region to couple to Gα. This may be important in understanding the signaling specificity of receptors in this subfamily and may provide insights into the mechanisms by which receptors activate different signal transduction pathways.
signaling pathways through such a small region? This question was raised again when in COS-7 cells we found that one residue in the N-terminal region of the PTH/PTHrP receptor was required and in oocytes two key residues appear to be needed for signal transduction through both pathways. Mutation of the positively charged lysine residue 382 to alanine markedly reduced the ability of the receptor to activate both adenylate cyclase and phospholipase C in COS-7 cells. In oocytes, two adjacent residues (threonine 381 and lysine 382) are critical for the dual activation of cAMP and IP production. The same two residues, therefore, are important for coupling to the different G-proteins mediating phospholipase C and adenylate cyclase activation.

The residues we identified as important in signal transduction are all clustered in a small contiguous region of the receptor. It appears unlikely that these few residues could contact two G-proteins at the same time. One possibility is that mutations cause a more global structural change in the receptor, which then disrupts the function of spatially separate sites that are responsible for interacting with distinct G-proteins. An alternate hypothesis is that dual signaling is achieved by the ability of different subpopulations of receptors to couple to different G-proteins. For example, one population of receptors couples to Gs, and at the same time, another population couples to a G-protein of the Gi family.

This latter hypothesis may explain the fact that all the mutant receptors we expressed retained high affinity binding. Previous studies from our group (33) and others (34) have shown that uncoupling of receptor-G-protein complexes results in complete loss of high affinity agonist binding. In these studies, we found that all mutant receptors tested, even those with marked uncoupling to Gi and Gs showed agonist binding and binding affinities comparable to WT receptors. The preservation of WT binding properties in these mutant receptors indicates the following. 1) The mutant receptors must be well expressed, and their levels of expression must be similar. 2) The ligand binding domain is not dramatically changed by these tandem- and single-alanine mutations. Therefore, the overall receptor structure is not likely to be dramatically altered. 3) The general physical interface between receptor and G-proteins must not be dramatically affected. Otherwise, the properties of the high affinity binding would be altered. Consistent with the hypothesis that there may be different populations of receptors to couple to specific G-proteins, those receptors with impaired coupling to one G-protein could probably couple productively to other G-proteins. This would assure that the ability of the receptor to bind PTH with high affinity is preserved.

In conclusion, our results suggest that the N-terminal region of the PTH/PTHrP receptor is critical for coupling to both Gs-adenylate cyclase and Gq-phospholipase C signal pathways. Discrete residues in this region participate in activation of Gi-adenylate cyclase and/or Gq-phospholipase C. Further studies will be necessary to define how these residues couple to specific G-proteins. Two of the residues we identified (Leu379 and Lys382) are highly conserved within the PTH/secretin receptor subfamily, and it will be of interest to whether these residues also mediate dual signal transduction in other members of this receptor subfamily.

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