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Domains of the parathyroid hormone (PTH) receptor required for regulation by G protein-coupled receptor kinases (GRKs)

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Abstract

To investigate the domains of the parathyroid hormone (PTH) receptor required for regulation by G protein-coupled receptor kinases (GRKs), we created mutant PTH receptors lacking potential GRK-phosphorylation sites. Mutant #1 was truncated at amino acid 544 and, therefore, lacked nine hydroxyl group-containing amino acids at the C-terminus. In mutant #2, we replaced threonines 392 and 399 in the third intracellular loop with glycines. Co-transfection of HEK293 cells with the wild-type receptor and either GRK2, GRK3, or GRK5 inhibited PTH-induced cyclic (cAMP) generation; co-transfection of GRK4 or GRK6 had no effect on PTH receptor responsiveness. GRK2-mediated inhibition of PTH receptor signaling was associated with enhanced phosphorylation receptor proteins. Co-expression of GRK2 similarly reduced PTH-induced cAMP generation by the wild-type receptor and mutant #1, and caused phosphorylation of receptor proteins to a similar extent. Co-expression of GRK2 had little effect on PTH-induced cAMP generation by mutant #2 but enhanced agonist-induced phosphorylation of mutant #2 compared with that of either the wild-type receptor or mutant #1. Enhanced phosphorylation of mutant #2 was associated with a reduction in agonist-induced internalization of mutant #2 compared with the wild-type receptor. Thus, phosphorylation of mutant #2 failed to cause receptor desensitization and inhibited receptor internalization. These data are consistent with the notion that: (a) GRKs contribute to regulating PTH receptor responsiveness, and (b) domains in the third intracellular loop are not required for agonist-induced phosphorylation of PTH receptors, but are critical for both agonist-induced internalization of PTH receptors and GRK2-mediated regulation of PTH receptor signaling.

Keywords: Parathyroid hormone; G protein-coupled receptor; Desensitization; G protein-coupled receptor kinase; Protein kinase A; Phospholipase C

1. Introduction

PTH plays a key role in regulating calcium and phosphate homeostasis [1]. Its effects are mediated by activating specific cell-surface receptors [2–4] and are subject to regulatory controls [5–10]. Prior exposure to PTH causes a reduction in receptor responsiveness [5–10]. This loss of receptor responsiveness or desensitization has been studied extensively in the cAMP-coupled β_2-adrenergic receptor and the phosphodiesterase-coupled receptor rhodopsin. In these receptor systems, agonist-specific desensitization is caused largely by direct phosphorylation of receptors at serine and threonine residues by general kinase systems such as protein kinase A (PKA) and PKC as well as by a family of receptor-specific kinases, GPCR kinases or GRKs [reviewed in Ref. 11]. Receptor phosphorylation is followed by binding of a second group of protein co-factors termed arrestins, which interfere with receptor–effector coupling presumably through steric mechanisms [12,13].

The receptor for PTH belongs to the large super-family of heptahelical GPCRs [2–4]. Its receptor binds both PTH and PTHrP with nanomolar affinities [2–4,14]. Activation of the PTH receptor by an agonist stimulates adenylyl cyclase and PLC [2–10] through G proteins belonging to the G_s and $G_{q/11}$ classes [15]. Previous studies suggest that agonist-specific desensitization of PTH receptors is associated with phosphorylation of receptor proteins [16,17]. More recently, two groups have presented evidence that: (a)

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Abbreviations: PTH, parathyroid hormone; PTHrP, PTH-related protein; cAMP, cyclic AMP; Tris, tris(hydroxymethyl)-aminomethane; K_d, dissociation constant; $B_{max,}$ maximal number of specific binding sites; PLC, phospholipase C; G-protein, guanine nucleotide regulatory protein; DMEM, Dulbecco's modified Eagle's medium; RT–PCR, reverse transcription–polymerase chain reaction; IBMX, isobutylmethyl xanthine; C-terminus, carboxyl-terminus; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; and D-PBS, Dulbecco's phosphate-buffered saline.

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PTH receptor phosphorylation is mediated, at least in part, by GRKs [18,19], and (b) agonist-induced phosphorylation of the receptor occurs on C-terminal domains [18,19]. At least some of these phosphorylation sites are found in the proximal portions of the C-terminus [18]. Truncation of the PTH receptor C-terminus to remove these phosphorylation domains attenuates PTH receptor phosphorylation but, surprisingly, has no effect on GRK-mediated inhibition of PTH receptor signaling [19]. While this observation may seem paradoxical based upon the current paradigm for receptor regulation [12,13], there is a precedent for such a finding in the literature. In *Drosophila*, truncation of the C-terminus of rhodopsin prevents light-induced phosphorylation of rhodopsin, but has no effect on desensitization [20]. Based upon this observation, it has been suggested that the C-terminus of rhodopsin contains auto-inhibitory domains that prevent arrestin binding [20]. Receptor phosphorylation causes a conformational change that exposes the arrestin binding sites and permits arrestin-induced desensitization. Deleting C-terminal domains of rhodopsin eliminates the need for receptor phosphorylation to expose the arrestin binding site, thus permitting phosphorylation-independent desensitization. Similar auto-inhibitory functions for the PTH receptor C-terminus could also explain the observation [19] that large C-terminal truncations of the PTH receptor permit agonist-induced inhibition of PTH receptor signaling in the absence of receptor phosphorylation.

To explain the dissociation between GRK-mediated inhibition of PTH receptor signaling and GRK-mediated phosphorylation [18,19], we made two hypotheses. First, the PTH receptor may be phosphorylated exclusively on the C-terminus, but phosphorylation proceeds in a hierarchical fashion, that is, phosphorylation of the previously identified proximal phosphorylation sites is required to induce phosphorylation of sites in the distal C-terminus. These distal C-terminal sites might be the critical sites for GRK-mediated regulation. Indeed, the proximal phosphorylation sites have motifs that are more likely to be phosphorylated by general kinase systems rather than GRKs [2,21]; in contrast, the distal C-terminus contains five hydroxyl group-containing amino acids that have favorable motifs for phosphorylation by GRKs [2,22]. In the second hypothesis, the PTH receptor may be phosphorylated by GRKs on domains outside the C-terminus. These alternative GRK phosphorylation sites might be the critical domains for promoting arrestin binding and, in turn, receptor desensitization. To test these hypotheses, we constructed two mutant receptors. Mutant #1 was truncated at amino acid 544. This mutant receptor retained the proximal C-terminal phosphorylation domains but lacked nine hydroxyl group-containing amino acids in the distal C-terminus, including five with favorable motifs for phosphorylation by GRKs [2,22]. We reasoned that this more conservative mutation would not only preserve the proximal phosphorylation sites, but might also preserve potential auto-inhibitory functions of the C-terminus and permit the role of GRK-induced phosphorylation of the PTH receptor to be appreciated. To investigate the role of potential GRK phosphorylation sites outside the C-terminus, we identified additional hydroxyl group-containing amino acids in the third intracellular loop with favorable GRK phosphorylation motifs [2,22]. Mutant #2 was created by replacing these hydroxyl group-containing amino acids (threonines 392 and 399) in the third intracellular loop with glycines. Using these mutant receptors, we investigated the role of PTH receptor phosphorylation in GRK-mediated regulation of PTH receptor signaling.

2. Materials and methods

2.1. Materials

Rat PTH(1–34) and human [^{125}I]-(Nle^{8,18},Tyr^{34})-PTH(1–34) amide were obtained from the Sigma Chemical Co. and Amersham Pharmacia, respectively. *Pfu* DNA polymerase was obtained from Stratagene. All PCR primers were prepared by Operon. Cell culture reagents were obtained from Gibco BRL, and [^{3}H]adenine was purchased from New England Nuclear. Human embryonic kidney (HEK293) cells were obtained from the American Type Culture Collection. The 12CA5 monoclonal antibody was purchased from Boehringer Mannheim. A rabbit polyclonal antibody against a 15 amino-acid sequence in the amino terminus of the rat PTH receptor was obtained from Covance. Antibodies to GRKs [23,24] were a gift from Dr. Robert J. Lefkowitz. The cDNAs for bovine GRK2, bovine GRK3, human GRK4, bovine GRK5, and human GRK6 were also a gift from Dr. Robert J. Lefkowitz and were produced as previously described [25].

2.2. Isolation and mutagenesis of a cDNA encoding the rat PTH receptor

A cDNA clone encoding the rat PTH receptor was isolated by RT–PCR using total RNA prepared from the rat osteoblast cell line ROS 17/2.8. This cell line expresses high levels of PTH receptor proteins [2]. PCR primers were prepared based upon the published sequences of the rat PTH receptor cDNA [2]. The primers were designed to create unique *Eco*RI and *Kpn*I restriction sites just 5′ to the start codon and just 3′ to the stop codon, respectively. The primer pairs encompassed nucleotides 43–96 (CCCCGAGGGACGC GGCCCTAGGAATTCGCGATGGGGGCCGCCCGGAT CGCACCC) and 1897–1868 (TCCATCTGTCCAGGTACC CAGGCCAGCAGT) of the rat PTH receptor cDNA [2]. The reverse transcription reaction was performed using Superscript reverse transcriptase (Gibco) and 2 μg of total cellular RNA prepared using the Trizol reagent (Gibco) according to the directions of the manufacturer. PCR was performed with the high fidelity, thermostable *Pfu* DNA polymerase to minimize mutations. After cutting with the appropriate restriction enzymes, the PCR product was li-

gated into the vector pBK-CMV(ΔlacZ) [26]. This vector is a derivative of pBK-CMV (Stratagene), which has been modified to create a short open reading frame encoding an amino terminal 12CA5 epitope [26,27]. By designing the PCR primers appropriately, we were able to insert our PCR product into the *Eco*RI restriction site in-frame with the sequences encoding the 12CA5 epitope, thus creating a 12CA5-tagged PTH receptor. Sequencing of the construct using the dideoxy method [28] confirmed that the PCR product was inserted in the proper orientation and in-frame.

To create mutant PTH receptors, we took advantage of a unique *Hin*dIII site in the proximal portions of the third intracellular loop [2] and the unique *Kpn*I site just distal to the stop codon. The C-terminal truncation mutant (mutant #1) was created using PCR to insert an in-frame stop codon at amino acid 544 and the primer pairs encompassing nucleotides 1226–1245 (TTGCCACTAAGCTTCGGGAG) and nucleotides 1728–1684 (AGTGACTGGTAGGGTAC CAGTCTCCTAGGCTGGAGCCCCTGGCTT) of the rat PTH receptor cDNA. To replace threonines 392 and 399 with glycines, we used PCR and the primer pairs encompassing nucleotides 1231–1281 (ACTAAGCTTCGGGAGGCCAAT GCGGGCCGGTGTGACGCCAGGCAGCAGTAC) and nucleotides 1897–1868 (TCCATCTGTCCAGGTACCCAG GCCAGCAGT) of the rat PTH receptor cDNA. PCR was performed with *Pfu* DNA polymerase and the rat PTH receptor cDNA as template. The PCR products were cut with *Hin*dIII and *Kpn*I and then were subcloned into the vector pBK-CMV(ΔlacZ) [26] containing the rat PTH receptor cDNA (see above). Mutant constructs were sequenced using the dideoxy method [28] to confirm the desired mutations.

As discussed above, our mutant and wild-type constructs were inserted into the pBK-CMV(ΔlacZ) vector [26] to create PTH receptors tagged at the amino terminus with the 12CA5 epitope [27]. PTH receptors tagged at the amino terminus with the 12CA5 epitope have been isolated successfully from COS-1 cells by immunoprecipitation [19]. However, we were unable to isolate our 12CA5-tagged PTH receptor constructs by immunoprecipitation from HEK293 cells perhaps because the PTH receptor contains an N-terminal signal peptide [2] that is cleaved prior to insertion of the receptor into the plasma membrane, thus removing our N-terminal epitope. We therefore chose to insert the 12CA5 epitope into the amino terminus after the putative signal peptide sequence. Using the technique of mutually priming oligonucleotides [29], we inserted the epitope between amino acids 89 and 90. This location was chosen because this portion of the receptor contains several amino acids common to the 12CA5 epitope sequence and facilitated design of the primer pairs. The initial PCR reactions were performed using the rat PTH receptor cDNA as template and primer pairs encompassing: (a) nucleotides 43–96 (CCCCGAGGGACGCGGCCCTAGGAATTCGCGATGG GGGCCGCCCGGATCGCACCC) and nucleotides 339–319 (AGGGTAGAACTTTCCCGATGC) and (b) nucleotides 325–357 (GGAAAGTTCTACCCTTACGACGTCCCA GACTACGCGTCTAAAGAGAACAAG) and nucleotides 1245–1226 (CTCCCGAAGCTTAGTGGCAA). The mutually priming PCR products were combined, and one cycle of PCR was performed prior to the addition of the primer pairs encompassing nucleotides 43–96 and nucleotides 1245–1226. All PCR reactions were performed with *Pfu* DNA polymerase. To insert the 12CA5-tagged sequence into wild-type or mutant receptors, we took advantage of the unique *Eco*RI site we had created in the N-terminus just 5′ to the start codon (see above) and a unique *Hin*dIII site in the rat PTH receptor cDNA [2]. After cutting the PCR product with *Eco*RI/*Hin*dIII, appropriately sized fragments were isolated from 1% agarose gels and subcloned into the pBK-CMV(ΔlacZ) vectors [26] containing the wild-type or mutant constructs. Sequencing of the constructs using the dideoxy method [27] confirmed that the 12CA5 epitope was inserted in the proper orientation and in-frame.

2.3. Culture and transfection of HEK-293 cells

HEK293 cells were grown and subcultured as previously described [30]. For transfection, HEK293 cells were plated in either 60-mm dishes or 6-well plastic culture dishes (9.5 cm^2/well) (Costar) and grown to approximately 80% confluency. Cells were then transfected using the calcium phosphate method [28]. To express PTH receptors, we used 2 μg of plasmid DNA/mL of transfection solution for wild-type receptors, mutant #1, and mutant #2. Preliminary experiments suggested that this amount of plasmid DNA optimized the level of receptor expression and transfection efficiency (≈20–40%). For each transfection, 1 mL of transfection solution was added drop-wise to a single 60-mm dish or to 2 wells of a 6-well plastic culture dish (9.5 cm^2/well). In the co-transfection experiments, GRK plasmid DNA was included in the transfection solution at a concentration of 1 μg/mL. Following an overnight incubation, the DNA solution was replaced with DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). HEK293 cells were studied 48 hr following transfection.

2.4. Radioligand binding studies

Radioligand binding studies were performed using cell monolayers grown in 96-well plastic culture dishes. For the binding assays, cells were transfected in 60-mm dishes as described above. The following day, transfected cells were plated at a density of 5×10^4/mL and then studied 48 hr following transfection. In the standard binding assay, cells were washed once with DMEM and then incubated in 40 μL of binding DMEM containing 0.1 to 20 nM human [^{125}I]-(Nle^{8,18}, Tyr^{34})-PTH(1–34) amide. After incubation for 1 hr at room temperature, cells were washed twice with D-PBS and then were solubilized in 200 μL of 0.1 N NaOH.

Non-specific binding was determined by measuring the amount of radioactivity bound in the presence of 1 μM unlabeled rat PTH(1–34). Radioactivity in the solubilized cell aliquots was quantitated by γ-counting. Equilibrium binding data were analyzed by the method of Scatchard [31] to give estimates of the B_{max} and apparent equilibrium K_d by fitting the data to a nonlinear model using the ENZFITTER computer program (Elsevier-Biosoft). Data are expressed as femtomoles per million cells.

2.5. Measurement of cAMP generation

Generation of cAMP was measured by the method of Salomon *et al.* [32]. For these studies, cells were plated at a density of 2–5 $\times$ 10^4/mL in 6-well plastic culture dishes (9.5 cm^2/well). After reaching approximately 80% confluence, cells were transfected as described above. Two days later, cells were changed to medium containing 2 μCi/mL of [^{3}H]adenine. After 90 min, cells were incubated for the indicated times with the agents to be tested or their vehicle in 1 mL of medium at 37° containing 100 μM IBMX. IBMX was included in the incubation medium to inhibit the breakdown of cAMP. The reaction was stopped by aspirating the medium, adding 1 mL of STOP solution (2.5% perchloric acid containing 100 μM cAMP and 1 μCi [^{14}C]cAMP), and placing the samples on ice. After 30 min, the STOP solution was transferred to a microcentrifuge tube, neutralized with 100 μL of 4.2 M KOH, and placed on ice. After 10 min, neutralized samples were centrifuged at 10,000 *g* for 5 min at 4°. Two hundred fifty microliters of supernatant was mixed with 800 μL of H_2O and applied to 1.0-mL columns packed with Dowex AG 50W-X4 (Bio-Rad). After discarding the flow through and a 2-mL H_2O wash, a 3-mL H_2O wash was collected, mixed with 200 μL of 1.5 M imidazole (pH 7.2), and applied to 1.0-mL columns of Alumina N type 1 (ICN). The column flow through and a 1-mL 0.1 M imidazole (pH 7.5) wash were collected and dissolved in 17 mL of Safety-Solve (Research Products International), and ^{3}H/^{14}C were quantitated by liquid scintillation counting. Data are expressed as the percent increase above basal cAMP generation.

2.6. Immunoblotting of GRKs

Expression of GRK2 and GRK3 was evaluated using a rabbit polyclonal antibody [23] that recognizes both GRKs. Expression of GRK4, GRK5, and GRK6 was evaluated using a mouse monoclonal antibody [24] that recognizes all three GRKs. Cells were solubilized in Laemmli sample buffer [33]. Proteins were separated on 12% polyacrylamide gels with 0.1% SDS as described by Laemmli [33]. Separated proteins were transferred from the polyacrylamide gels to nitrocellulose (Bio-Rad) by using a Milliblot^{TM} semi-dry transfer apparatus (Millipore) according to the recommendations of the manufacturer. The nitrocellulose was blocked for 1 hr in Tris–saline buffer (20 mM Tris–HCl, 137 mM NaCl, pH 7.6) (TBS) with 0.2% Tween 20 (T-TBS) and 5% non-fat dry milk (BLOTTO). After blocking, the primary antibody was added at a dilution of 1:2000 in BLOTTO. The blot was incubated at room temperature for 1 hr with gentle rocking followed by three washes with T-TBS. The horseradish peroxidase-labeled secondary antibody (Amersham) was added at a dilution of 1:2000 in BLOTTO. After rocking for 1 hr at room temperature, the blot was washed once with T-TBS and twice with TBS. Proteins were detected by enhanced chemiluminescence (ECL) according to the specifications of the manufacturer (Amersham).

2.7. Immunoprecipitation of epitope-tagged PTH receptors

Forty-eight hours following transfection, cells were washed with phosphate-free DMEM (Gibco) and then were placed in phosphate-free DMEM containing 0.1 to 0.2 mCi ^{32}P (New England Nuclear). After 90 min, cells were stimulated with agonist for 10 min at the indicated concentrations of agonist or vehicle. The reaction was stopped by washing the cells with ice-cold D-PBS and then scraping the cells into 1 mL of ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 8.0), 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 100 nM calyculin, 1 μg/mL of leupeptin, 1 μg/mL of pepstatin, 1 μg/mL of aprotinin, and 100 μg/mL of phenylmethylsulfonyl fluoride (PMSF). The lysate was transferred to a 1.5-mL microcentrifuge tube and then rocked for 30 min at 4°. Insoluble material was removed by centrifugation at 10,000 *g* for 4 min at 4°. One milliliter of supernatant was transferred to a 1.5-mL microcentrifuge tube, and 10 μg of 12CA5 monoclonal antibody (Boehringer Mannheim) was added. After rocking at 4° for 1 hr, 60 μL of Protein G PLUS/Protein A-agarose solution (Oncogene) was added, and the samples were rocked at 4° for 1 hr. The Protein G PLUS/Protein A-agarose was washed three times in lysis buffer. SDS-sample buffer (100 μL) was added to the pellet and boiled for 10 min. Proteins were separated on 9% polyacrylamide gels with 0.1% SDS as described by Laemmli [33]. After drying the gels, phosphorylated proteins were detected by autoradiography.

2.8. Internalization of PTH receptor

PTH receptor internalization was determined by flow cytometry using a protocol modified from Barak *et al.* [34]. For the studies, 12CA5-tagged PTH receptors were transiently transfected into HEK293 cells. Two days following transfection, cells were harvested using 0.25% trypsin with 1 mM EDTA (Gibco) and then washed three times in room temperature D-PBS containing 2% fetal bovine serum (FBS). Cell suspensions were treated with PTH (100 nM) or

vehicle for the indicated times at room temperature. The reaction was stopped by washing with ice-cold D-PBS containing 2% fetal bovine serum (D-PBS/FBS). After washing, the cells were resuspended in 1 mL of ice-cold D-PBS/FBS containing either 1 μg/mL of the 12CA5 monoclonal antibody or a 1:100 dilution of a rabbit polyclonal antibody against the rat PTH receptor. The 12CA5 antibody was used to study internalization of epitope-tagged PTH receptors, and the rabbit polyclonal antibody was used to study internalization of PTH receptors lacking the 12CA5 epitope. After incubating the cell suspension for 30 min at 4°, the cells were washed three times with ice-cold D-PBS/FBS. Then the cells were resuspended in 1 mL of ice-cold D-PBS/FBS containing either a 1:2000 dilution of fluorescein-conjugated anti-mouse IgG (ICN Biomedicals) or a 1:1000 dilution of fluorescein-conjugated anti-rabbit IgG (Sigma). Then the suspensions were incubated for 30 min at 4°. After washing three times in ice-cold D-PBS, the cells were fixed in D-PBS containing 2% formalin. Flow cytometric analysis was performed within the Flow Cytometry Facility at Duke University Medical Center. Receptor internalization was defined as the percentage of cell surface receptors on vehicle-treated cells that were no longer accessible to antibodies outside the cells after treatment with 100 nM PTH. Baseline fluorescence from cells not transfected with the 12CA5-tagged PTH receptor was subtracted from each sample. In preliminary experiments, we found that there was a direct correlation between the B_{max} obtained by Scatchard analysis and the difference between the total fluorescence from cells transfected with the 12CA5-tagged PTH receptor minus the baseline fluorescence. To determine if the addition of PTH interfered with binding of the 12CA5 antibody, we treated cells with PTH(1–34) or its vehicle at 4° to prevent receptor internalization. In these experiments, the total fluorescence minus the baseline fluorescence in cells transfected with the PTH receptor and treated with vehicle was similar to that of cells transfected with the PTH receptor and treated with 100 nM PTH(1–34). These data suggest that the addition of PTH(1–34) did not interfere with binding of the 12CA5 antibody. Internalization of the PTH receptor lacking the 12CA5 epitope was similar to that of epitope-tagged PTH receptors at 5 min (52 ± 6% [wild type] vs 46 ± 7% [epitope tag]; P = NS) and at 10 min (64 ± 7% [wild type] vs 59 ± 8% [epitope tag]; P = NS) after the addition of PTH(1–34).

2.9. Statistical analysis

Data are presented as means ± SEM. For comparisons between two groups, statistical significance was assessed using an unpaired *t*-test. For comparisons between more than two groups, statistical analysis was performed by analysis of variance followed by Bonferroni's procedure for multiple pair-wise comparisons [35].

Table 1
Binding of [^{125}]PTH to wild-type or mutant PTH receptors

	B_{max} (fmol/million cells)	K_d (nM)
Wild-type	213 ± 20	1.8 ± 0.5
Mutant #1	182 ± 10	1.3 ± 01.2
Mutant #2	194 ± 15	1.4 ± 0.5

Data were analyzed by the method of Scatchard [31] to obtain the B_{max} and equilibrium K_d, and are presented as the means ± SEM of 3 assays.

3. Results

3.1. Effect of PTH receptor mutations on binding of [^{125}I]PTH(1–34)

We used PCR to create two mutant PTH receptors as described in "Materials and methods." Mutant #1 was truncated at amino acid 544 and, therefore, lacked nine hydroxyl group-containing amino acids in the distal C-terminus but retained the phosphorylation sites in more proximal portions of the C-terminus [2,18]. In mutant #2, we replaced threonines 392 and 399 in the third intracellular loop with glycines. Plasmids containing either the wild-type or mutant constructs were transfected into HEK293 cells. As shown in Table 1, the wild-type receptor was expressed at 213 ± 20 fmol/million cells with a K_d of 1.8 ± 0.5 nM. Both mutant PTH receptors were expressed at slightly lower levels and had slightly higher affinities for PTH (Table 1); however, these differences did not reach statistical significance.

3.2. Effect of GRKs on PTH receptor responsiveness

To investigate the role of GRKs in modulating PTH receptor responsiveness, we co-expressed the wild-type PTH receptor and either individual GRKs or empty vector in HEK293 cells. As shown in Fig. 1A, co-expression of either GRK2 or GRK3 inhibited PTH receptor responsiveness compared with cells co-transfected with empty vector. Fig. 1B shows that we obtained high levels of expression of GRK2 and GRK3 in our cell system. Co-transfection of either GRK2 or GRK3 had no significant effect on receptor expression or binding (Table 2). These data are consistent with the notion that both GRK2 and GRK3 are capable of regulating PTH receptor responsiveness.

We next investigated the effects of GRK4, GRK5, and GRK6 on PTH receptor responsiveness. For these experiments, we measured PTH-induced cAMP generation by cells co-transfected with the PTH receptor and either empty vector, GRK4, GRK5, or GRK6. As seen in Fig. 2A, co-transfection of GRK5 caused a decrease in PTH receptor responsiveness compared with cells co-transfected with vector. However, this difference did not reach statistical significance. Co-transfection of either GRK4 or GRK6 had no effect on PTH receptor responsiveness. Fig. 2B shows that we obtained high levels of expression of GRK4, GRK5,

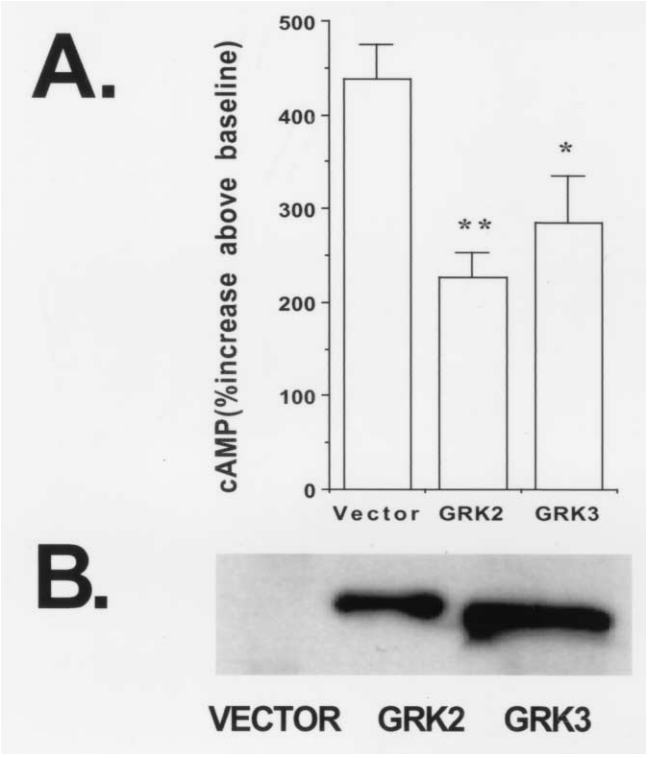

Fig. 1. Effects of GRK2 and GRK3 on PTH receptor responsiveness. HEK293 cells were co-transfected with the PTH receptor and either empty vector, GRK2, or GRK3. Two days following the transfection, cells were stimulated with 100 nM PTH(1–34) for 10 min, and then cAMP generation was measured as described in "Materials and methods." In parallel experiments, expression of GRK2 and GRK3 was assessed by immunoblotting using an antibody that recognizes both GRKs as described in "Materials and methods." As shown in panel A, the co-transfection of both GRK2 and GRK3 significantly reduced PTH-induced cAMP accumulation compared with that of the cells co-transfected with empty vector (*$P < 0.05$ or **$P < 0.025$ vs empty vector). Panel B shows the expression of GRK2 and GRK3 in our cell system. Basal cAMP generation rates were 236 ± 12, 212 ± 14, and 222 ± 16 cpm/well/10 min by cells co-transfected with vector, GRK2, and GRK3, respectively. Data are the means ± SEM of 8 experiments.

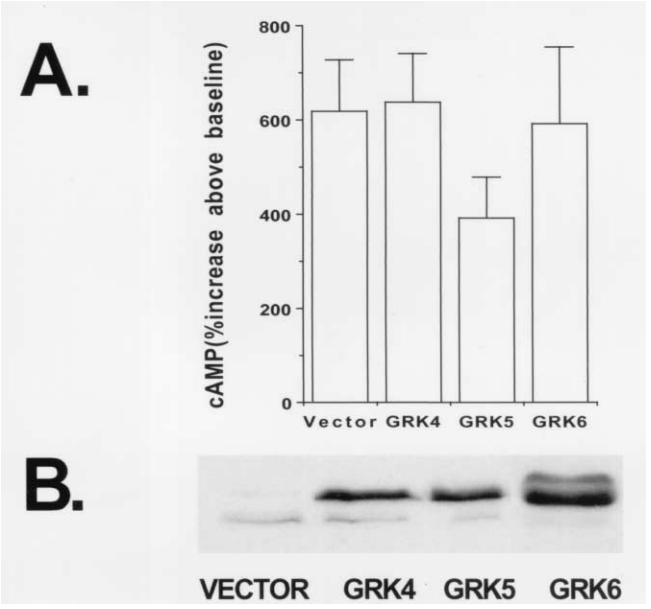

Fig. 2. Effects of GRK4, GRK5, and GRK6 on PTH receptor responsiveness. HEK293 cells were co-transfected with the PTH receptor and either empty vector, GRK4, GRK5, or GRK6. Two days following the transfection, cells were stimulated with 100 nM PTH(1–34) for 10 min, and then cAMP generation was measured as described in "Materials and methods." In parallel experiments, the expression of GRK4, GRK5, and GRK6 was assessed by immunoblotting using an antibody that recognizes all three GRKs, as described in "Materials and methods." Panel A shows the effect of co-expressing GRK4, GRK5, or GRK6 on PTH-stimulated cAMP accumulation. Basal cAMP generation rates were 266 ± 15, 244 ± 17, 231 ± 12, and 277 ± 18 cpm/well/10 min by cells co-transfected with vector, GRK4, GRK5, and GRK6, respectively. Data are presented as the means ± SEM of 4 experiments. Panel B shows the expression of GRK4, GRK5, and GRK6 in our cell system.

and GRK6 in our cell system. These data, taken together with the results shown in Fig. 1, are consistent with the notion that GRK2, GRK3, and possibly GRK5 are capable of modulating PTH receptor responsiveness.

In separate experiments, we determined the effects of GRK2 and GRK3 on PTH receptor responsiveness to various concentrations of PTH. Results of these studies are presented in Fig. 3. Co-transfection of the PTH receptor and either GRK2 or GRK3 both reduced maximal cAMP generation (V_{max}) and shifted the EC_{50} to the right. The V_{max} was 48% for GRK2 and 62% for GRK3. The EC_{50} values shifted from 0.95 to 1.2 and 1.4 nM for GRK3 and GRK2, respectively.

Table 2
Effect of co-transfecting GRK2 or GRK3 on PTH receptor binding

Co-transfection	B_{max} (fmol/million cells)	K_d (nM)
Vector	202 ± 18	1.7 ± 0.4
GRK2	192 ± 12	1.6 ± 1.0
GRK3	211 ± 16	1.8 ± 0.6

Data were analyzed by the method of Scatchard [31] to obtain the B_{max} and equilibrium K_d, and are presented as the means ± SEM of 3 assays.

3.3. Attenuation of GRK2-mediated regulation of PTH receptor responsiveness by the substitution of glycines for threonines 392 and 399

We next investigated the effect of our mutations on the regulation of PTH receptor responsiveness by GRKs. For these studies, we co-transfected HEK293 cells with the wild-type or mutant receptors and either GRK2 or its empty vector. As seen in Fig. 4, co-transfection of GRK2 similarly reduced PTH-induced cAMP generation by the wild-type receptors and the C-terminal truncation mutant (mutant #1). In contrast, co-transfection of GRK2 had little effect on PTH-induced cAMP generation by the third loop mutant (mutant #2). Basal cAMP generation rates were similar in wild-type receptors (240 ± 10 cpm/well/10 min), mutant #1 (255 ± 10 cpm/well/10 min), and mutant #2 (275 ± 17 cpm/well/10 min). Thus, GRK2-mediated inhibition of PTH

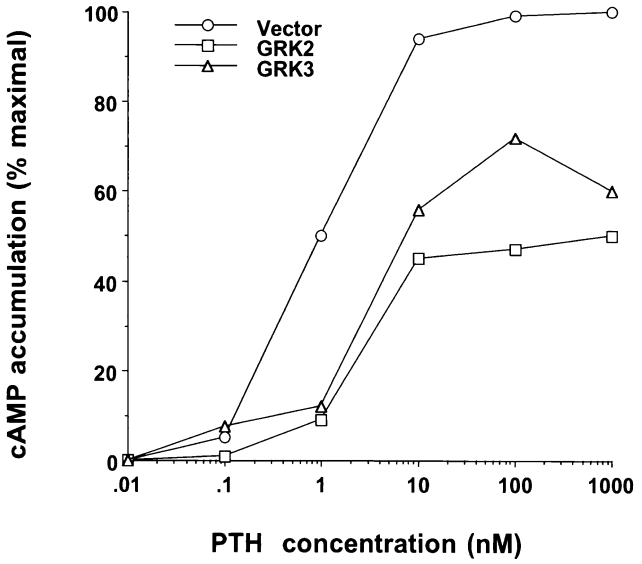

Fig. 3. Effects of GRK2 and GRK3 and PTH receptor responsiveness to various concentrations of PTH. HEK293 cells were co-transfected with the PTH receptor and either empty vector, GRK2, or GRK3. Two days following the transfection, cells were stimulated with the indicated concentrations of PTH(1–34) for 10 min, and then cAMP generation was measured as described in "Materials and methods." Data are expressed as a percentage of maximal cAMP production determined using 1 μM PTH(1–34). Maximal cAMP generation (V_{max}) was 48% for cells co-transfected with GRK2 and 62% for cells co-transfected with GRK3. The EC_{50} values were 0.95, 1.4, and 1.2 nM for cells co-transfected with vector, GRK2, and GRK3, respectively. Data are the means of 4 experiments.

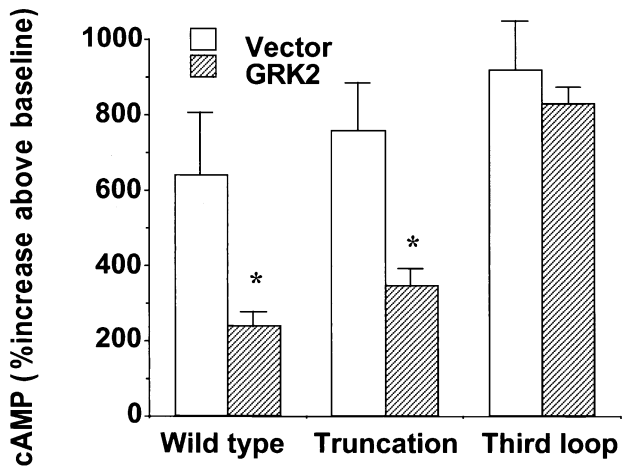

Fig. 4. Effect of PTH receptor mutations on GRK-mediated inhibition of PTH receptor signaling. HEK293 cells were co-transfected with wild-type or mutant receptors and either GRK2 or its empty vector. Two days following transfection, cells were stimulated with 100 nM PTH(1–34) for 10 min, and then cAMP generation was measured as described in "Materials and methods." Co-transfection of GRK2 significantly reduced PTH-induced cAMP generation by the wild-type receptors and the C-terminal truncation mutant (mutant #1). Co-transfection of GRK2 did not significantly affect PTH-induced cAMP generation by the third loop mutant (mutant #2). Basal cAMP generation rates were 240 ± 10, 255 ± 10, and 275 ± 17 cpm/well/10 min by wild-type receptors, mutant #1, and mutant #2, respectively. Data are the means ± SEM of 5 experiments. Key: (*) $P < 0.05$ vs empty vector.

receptor responsiveness is not affected by the truncation of C-terminal domains but, instead, is attenuated by deleting two potential GRK phosphorylation sites in the third intracellular loop. These data suggest that domains in the third intracellular loop are critical for GRK-mediated regulation of PTH receptor signaling.

3.4. Agonist-induced phosphorylation of wild-type and mutant PTH receptors

We next determined if our mutations affected agonist-induced phosphorylation of PTH receptor proteins. For these studies, we used PCR to insert the 12CA5 epitope at an internal site in the N-terminus as described in "Materials and methods." Radioligand binding studies suggested that insertion of the 12CA5 epitope in the N-terminus did not significantly affect the affinity of [^{125}I]PTH binding (K_d 1.6 ± 0.5 nM [wild-type] vs 1.9 ± 0.4 nM [12CA5 epitope]; P = NS) or cell surface expression of the PTH receptor (B_{max} 202 ± 20 fmol/million cells [wild-type] vs 190 ± 22 fmol/million cells [12CA5 epitope]; P = NS). Moreover, there was no effect of the epitope on agonist-induced cAMP generation (484 ± 62% above baseline [wild-type] vs 454 ± 35% above baseline [12CA5 epitope]; P = NS). To determine if our new 12CA5-tagged receptor could be used to monitor agonist-induced phosphorylation of the PTH receptor, HEK293 cells were transfected with either the epitope-tagged construct or a wild-type construct. Two days following transfection, cells were loaded with ^{32}P and then stimulated with 100 nM PTH(1–34) or vehicle. After 10 min, cells were harvested, and immunoprecipitation was performed as described in "Materials and methods." As shown in Fig. 5, stimulation of the transfected HEK293 cells with 100 nM PTH(1–34) caused phosphorylation of a broad band at ≈80 kDa. The band was not seen in cells transfected with the wild-type receptor, suggesting that the ≈80 kDa band represented the phosphorylated PTH receptor. These data suggest that this new construct is suitable for studying agonist-induced phosphorylation of the PTH receptor.

We next determined the effect of co-expressing GRK2 on agonist-induced phosphorylation of PTH receptors. For these studies, HEK293 cells were transfected with the wild-type PTH receptor and either GRK2 or its empty vector. Two days following transfection, cells were loaded with ^{32}P and then stimulated with 100 nM PTH(1–34) or its vehicle. After 10 min, cells were harvested, and PTH receptors were immunoprecipitated as described in "Materials and methods." Results of these experiments are shown in Fig. 6. Similar to results reported by other investigators [18], co-transfection of GRK2 caused an approximately 2- to 3-fold increase in agonist-induced phosphorylation of the PTH receptor without altering its basal phosphorylation state.

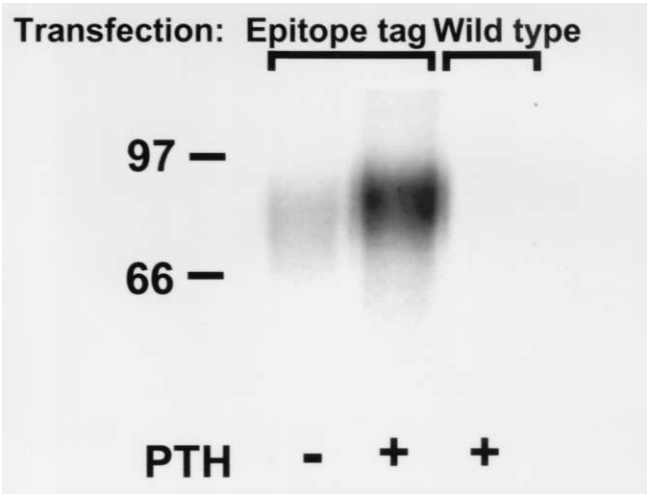

Fig. 5. Agonist-induced phosphorylation of PTH receptor proteins. HEK293 cells were transfected with either the wild-type PTH receptor or a construct encoding a 12CA5-tagged PTH receptor. Two days following transfection, cells were loaded with ^{32}P and then were stimulated with 100 nM PTH(1–34) or vehicle. After 10 min, cells were harvested, and immunoprecipitation of receptor proteins was performed as described in "Materials and methods." The phosphorylation state of receptor proteins was assessed by autoradiography after separation on 9% polyacrylamide gels. Apparent molecular mass is indicated in kDa. Stimulation of HEK293 cells transfected with the epitope-tagged receptor caused phosphorylation of a broad band of ≈80 kDa. This band was not seen in cells transfected with the wild-type receptor. Similar results were obtained in 2 separate studies.

To determine if our mutations affected agonist-induced phosphorylation of PTH receptors, HEK293 cells were transfected with GRK2 and either wild-type or mutant PTH receptors that had been tagged with 12CA5 epitope. Two days following transfections, cells were loaded with ^{32}P and then stimulated with 100 nM PTH(1–34) or vehicle. After 10 min, cells were harvested, and PTH receptors were immunoprecipitated as described in "Materials and methods." Results of these experiments are shown in Fig. 7. The treatment of cells expressing wild-type receptors induced phosphorylation of a broad band of ≈80 kDa. Stimulation of cells expressing the truncation mutant (mutant #1) caused phosphorylation of a broad band of ≈70 kDa. As shown in Table 3, densitometric analysis suggested that the level of phosphorylation of the truncation mutant was similar to that of the wild-type receptor. These data are consistent with previously published studies [18,19,36] and indicate that the predominant PTH receptor phosphorylation sites are not in the distal C-terminus. In contrast, the absence of threonines 392 and 399 in the third intracellular loop (mutant #2) enhanced agonist-induced phosphorylation of PTH receptor proteins compared with the wild-type receptor or mutant #1 (Table 3). These data suggest that domains in the third

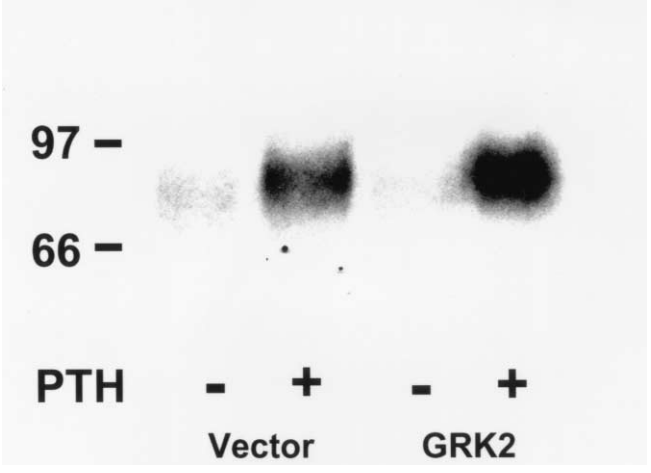

Fig. 6. Effect of GRK2 on agonist-induced phosphorylation of PTH receptors. HEK293 cells were transfected with the wild-type PTH receptor and either GRK2 or its empty vector. Two days following transfection, cells were loaded with ^{32}P and then were stimulated with 100 nM PTH(1–34) or vehicle. After 10 min, cells were harvested, and PTH receptors were immunoprecipitated as described in "Materials and methods." Co-transfection of GRK2 caused an approximately 2- to 3-fold increase in agonist-induced phosphorylation of the PTH receptor. Apparent molecular mass is indicated in kDa. Similar results were obtained in 3 separate studies.

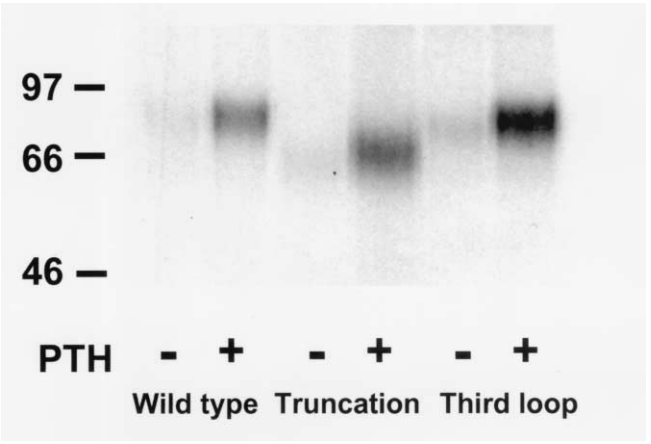

Fig. 7. Agonist-induced phosphorylation of wild-type and mutant PTH receptors in cells overexpressing GRK2. HEK293 cells were co-transfected with GRK2 and either wild-type or mutant PTH receptors that had been tagged with 12CA5 epitope. Two days following transfections, cells were loaded with ^{32}P and then were stimulated with 100 nM PTH(1–34) or vehicle. After 10 min, cells were harvested, and PTH receptors were immunoprecipitated as described in "Materials and methods." The phosphorylation state of receptor proteins was assessed by autoradiography after separation on 9% polyacrylamide gels. Agonist-induced phosphorylation of the third loop mutant (mutant #2) was enhanced significantly ($P < 0.025$) compared with that of the wild-type receptor or the C-terminal truncation mutant (mutant #1). Apparent molecular mass is indicated in kDa. Similar results were obtained in 7 separate studies.

Table 3
Phosphorylation of mutant and wild-type PTH receptors

	Densitometry units		
	Wild-type	Mutant #1	Mutant #2
Basal	25,632 ± 3,967	22,139 ± 2,551	29,407 ± 3,653
Agonist-induced	113,493 ± 2,258	109,718 ± 10,113	167,461 ± 6,641*

Data are presented as the means ± SEM Of 7 assays.
** $P < 0.025$ vs wild-type and mutant #1.

intracellular loop are not required for agonist-induced phosphorylation of the PTH receptor.

3.5. Attenuation of agonist-induced internalization of PTH receptors by the substitution of glycines for threonines 392 and 399

To determine if the absence of threonines 392 and 399 affected internalization of PTH receptors, we co-transfected cells with the wild-type PTH receptor or mutant #2 and either GRK2 or empty vector. Two days following transfection, we assessed PTH-induced internalization of receptor proteins as described in "Materials and methods." Results of these studies are shown in Fig. 8. In the absence of GRK2 overexpression (panel A), substitution of glycines for threonines at amino acids 392 and 399 significantly decreased internalization of PTH receptors at the 10-min time point. In cells overexpressing GRK2 (panel B), internalization of the mutant PTH receptor was decreased at both 5 and 10 min following the addition of 100 nM PTH(1–34). Comparison of the rates of internalization suggested that the internalization rate tended to be increased in cells co-transfected with GRK2 (panel B) compared with cells transfected with empty vector (panel A); however, this difference was not statistically significant. These data suggest that substitution of glycines for threonines 392 and 399 attenuates agonist-induced internalization of PTH receptors.

4. Discussion

The biological effects of PTH are mediated by binding to specific cell surface GPCRs [2–4] and are subject to regulatory controls [5–10]. In the continuous presence of an agonist, the effects of PTH are attenuated [5–10]. This desensitization is associated with phosphorylation of receptor proteins [16–19]. Recent studies suggest that PTH receptor phosphorylation is mediated, at least in part, by a family of receptor specific kinases or GRKs [18,19]. In the present studies, we found that co-transfection of the PTH receptor and individual GRK family members inhibited PTH-induced cAMP generation with a rank order potency of GRK2>GRK3>GRK5. Co-expression of GRK4 or GRK6 had little effect on PTH receptor responsiveness. GRK2-mediated regulation of PTH receptor signaling was associated with enhanced phosphorylation receptor proteins and was not affected by truncation of the C-terminus at amino acid 544 (mutant #1). In contrast, the absence of threonines 392 and 399 in the third intracellular loop (mutant #2) largely abolished the regulation of PTH receptor responsiveness by GRK2. This attenuation of GRK2-mediated regulation was associated with: (a) enhanced agonist-dependent phosphorylation of mutant #2 compared with either the wild-type receptor or mutant #1, and (b) inhibition of agonist-induced internalization of mutant #2 compared with the wild-type receptor. These data suggest that threonines 392 and 399 in the third intracellular loop are not required for agonist-dependent phosphorylation of PTH receptor proteins; however, domains in the third intracellular loop are critical for both agonist-induced internalization of PTH receptors as well as GRK2-mediated regulation of PTH receptor signaling.

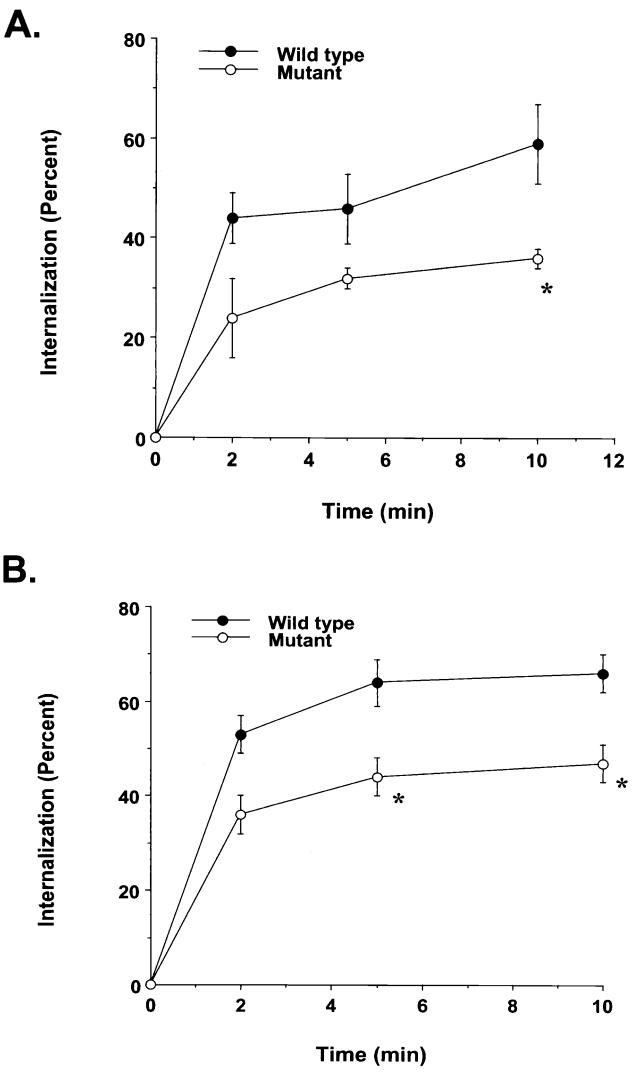

Fig. 8. Agonist-induced internalization of wild-type PTH receptors and mutant #2. HEK293 cells were transfected with the wild-type PTH receptor or mutant #2 and either empty vector (panel A) or GRK2 (panel B). Two days following transfection, cells were stimulated with 100 nM PTH(1–34) for the indicated times, and then PTH-induced internalization of receptors was determined as described in "Materials and methods." As shown in panel A, in the absence of GRK2 overexpression, internalization of mutant #2 was significantly attenuated compared with the wild-type receptor at the 10-min time point. In cells overexpressing GRK2, substitution of glycines for threonines 392 and 399 in the third intracellular loop (mutant #2) significantly attenuated internalization of PTH receptors at both the 5- and 10-min time points. Data are the means ± SEM of 4 experiments. Key: (*) $P < 0.05$ vs the wild-type.

Since phosphorylation of the third loop mutant (mutant #2) did not attenuate PTH receptor signaling, it is likely that substitution of glycines for threonines 392 and 399 in the third intracellular loop affected a mechanism required for

receptor regulation following the phosphorylation reaction. Based upon the current concepts of GPCR regulation [11–13], phosphorylation of receptor proteins is followed by binding of a second group of protein co-factors termed arrestins, which desensitize receptor signaling presumably by sterically interfering with receptor–effector coupling [12,13]. While the mechanisms of phosphorylation-induced arrestin binding are not known with certainty, it has been postulated that phosphorylation promotes arrestin binding by either: (a) providing additional determinants required for binding of arrestin to the receptor, or (b) causing a conformation in the receptor that exposes the arrestin binding sites [12]. In support of this latter hypothesis, rhodopsin is phosphorylated in an agonist-dependent fashion exclusively on the C-terminus [20]; yet, the interaction of visual arrestin with rhodopsin can be inhibited by synthetic peptides representing the first and third intracellular loops [12,37]. Thus, domains of rhodopsin, different from the phosphorylation domains, appear to compete with rhodopsin for arrestin binding. By deleting threonines 392 and 399 in the third intracellular loop, it is possible that arrestin binding was affected by one or both of these postulated mechanisms. A failure to promote high-affinity arrestin binding might also explain the apparent enhancement of agonist-induced phosphorylation of mutant #2. Indeed, in other receptor systems, arrestins are critical for receptor internalization and dephosphorylation followed by recycling of receptor proteins [38,39]. If this dephosphorylation and recycling mechanism is disrupted, agonist-induced phosphorylation of PTH receptors might be enhanced. In support of this hypothesis, inhibition of GPCR internalization increases the phosphorylation state of the β-adrenergic receptor following agonist stimulation [40].

To determine if the absence of threonines 392 and 399 affected internalization of PTH receptors, we compared the extent of agonist-induced internalization in HEK293 cells transfected with the wild-type receptor to that of cells transfected with mutant #2. We found that substitution of glycines for threonines 392 and 399 in the third intracellular loop decreased agonist-induced internalization of PTH receptor proteins by approximately 30%. Thus, enhanced agonist-dependent phosphorylation of mutant #2 is associated with attenuation of receptor internalization. These data suggest that domains in the third intracellular loop contribute to internalization of PTH receptors. Moreover, these data support the notion that attenuation of receptor internalization leads to enhanced phosphorylation of the PTH receptor, possibly by inhibited dephosphorylation and recycling of receptor proteins.

Previous studies have suggested [19] that a truncation of all but the most proximal portions of the C-terminus largely prevents agonist-induced phosphorylation of the PTH receptor without affecting either binding of GRKs to the receptor or GRK-mediated inhibition of receptor signaling. These authors hypothesized that signaling by the PTH receptor is inhibited by the first step in agonist-induced desensitization, that is, binding of GRKs to the GPCR prior to the phosphorylation reaction. An alternative explanation has been proposed for phosphorylation-deficient mutants of rhodopsin. In this receptor system, truncation of the C-terminus abolishes light-induced phosphorylation of rhodopsin but not desensitization [20]. Investigators have proposed that the C-terminus of rhodopsin contains auto-inhibitory domains that prevent arrestin binding [20]. Receptor phosphorylation causes a conformational change that exposes the arrestin binding sites and permits arrestin-induced desensitization, whereas deleting C-terminal auto-inhibitory domains permits arrestin binding in the absence of rhodopsin phosphorylation. Further studies will be necessary to test these hypotheses. However, these data suggest that receptor phosphorylation is not an absolute requirement for desensitization. Conversely, our findings using the third loop mutant (mutant #2) indicate that GPCR phosphorylation does not necessarily result in desensitization. This dissociation between receptor phosphorylation and attenuation of receptor responsiveness is consistent with the notion that desensitization requires multiple steps that may be differentially affected by the experimental manipulation.

Nissenson and coworkers [18] found that the PTH receptor is phosphorylated on hydroxyl group-containing amino acids in the proximal portions of the C-terminus. Phosphoamino acid analysis of agonist-phosphorylated PTH receptors further indicated that the receptor is phosphorylated exclusively on serine residues [18]. This latter finding suggests that substituting glycines for threonines 392 and 399 in the third intracellular loop inhibited GRK-mediated regulation by mechanisms that are not likely to involve direct phosphorylation of receptor proteins. Our findings using mutant #1 are also consistent with the notion that PTH receptor phosphorylation occurs on serine residues in the proximal C-terminus. Indeed, truncation of the PTH receptor at more distal domains of the C-terminus (mutant #1) had little effect on agonist-induced phosphorylation. This lack of effect on agonist-induced phosphorylation indicates that the PTH receptor is not phosphorylated in a hierarchical fashion as initially hypothesized. If phosphorylation of proximal C-terminal domains were required for phosphorylation of more distal sites, then agonist-induced phosphorylation of mutant #1 would be decreased compared with phosphorylation of the wild-type receptor. While it is possible that the sensitivity of the experimental technique was unable to detect subtle differences in PTH receptor phosphorylation, the present data are most consistent with phosphorylation of the PTH receptor exclusively on proximal portions of the C-terminus.

Previous studies indicate that co-transfection of the human PTH receptor and either GRK2, GRK3, or GRK5 potently inhibits PTH-induced inositol phosphatase generation in COS-1 cells with a rank order potency of GRK2>GRK3>GRK5 [19]. This same group reported that co-transfection of the PTH receptor and GRK2 caused a modest reduction in PTH-stimulated cAMP production

[19]. In the present studies, we found that co-transfection of the PTH receptor and either GRK2 or GRK3 significantly attenuated cAMP generation following PTH receptor activation. We observed a more modest effect following co-transfection of GRK5. In contrast, co-transfection of GRK4 or GRK6 had no significant effect on PTH-induced cAMP production. Taken together, these observations suggest that GRK2>GRK3>GRK5 inhibits second messenger generation by the PTH receptor. Because GRK2, GRK3, and GRK5 are widely expressed [40], it is likely that these GRKs play key roles in regulating PTH receptor responsiveness. In this regard, recent studies by Fukayama *et al.* [41] found that a dominant negative mutant of GRK2 attenuated PTH receptor desensitization in an osteosarcoma cell line, suggesting that GRKs regulate PTH signaling in osteoblasts.

Truncation of PTH receptor C-terminal domains has been reported to either enhance [42] or have no effect [43] on the affinity of PTH binding as well as to either enhance [19,42] or have no effect [42] on second messenger generation. We found that the absence of the distal C-terminus did not significantly affect PTH binding or PTH-induced cAMP generation. Similarly, we found that the absence of threonines 392 and 399 did not significantly alter ligand binding or cAMP production. The lack of effect of our mutations on PTH receptor binding or cAMP generation may be related to the more conservative mutations used in the present study compared with previously published work [19,42,43]. Based on the present data, it is therefore unlikely that either the distal C-terminus or threonines 392 and 399 play prominent roles in PTH binding or receptor–effector coupling.

In summary, we found that GRK2, GRK3, and possibly GRK5 inhibited agonist-induced cAMP generation by the PTH receptor. This regulatory effect requires the presence of threonines 392 and 399 in the third intracellular loop; however, the absence of these threonine residues does not prevent agonist-induced phosphorylation of PTH receptor proteins. Indeed, substitution of glycines for threonines 392 and 399 enhanced agonist-induced phosphorylation of mutant PTH receptors compared with the wild type. This enhanced phosphorylation of mutant PTH receptor proteins was associated with inhibition of PTH receptor internalization. These data suggest that domains in the third intracellular loop are not required for agonist-induced phosphorylation of PTH receptors, but are critical for both agonist-induced internalization of PTH receptors and GRK2-mediated regulation of PTH receptor signaling.

Acknowledgments

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