Eliminating phosphorylation sites of the parathyroid hormone receptor type 1 differentially affects stimulation of phospholipase C and receptor internalization

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Submitted 19 January 2008; accepted in final form 19 June 2008

The parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor (PTH1R) belongs to family B of seven-transmembrane-spanning receptors and is activated by PTH and PTHrP. Upon PTH stimulation, the rat PTH1R becomes phosphorylated at seven serine residues. Elimination of all PTH1R phosphorylation sites results in prolonged cAMP accumulation and impaired internalization in stably transfected LLC-PK1 cells. The present study explores the role of individual PTH1R phosphorylation sites in PTH1R signaling through phospholipase C, agonist-dependent receptor internalization, and regulation by G protein-coupled receptor kinases. By means of transiently transfected COS-7 cells, we demonstrate that the phosphorylation-deficient (pd) PTH1R confers dramatically enhanced coupling to Gs₁₁₁ proteins upon PTH stimulation predominantly caused by elimination of Ser⁴⁹¹/⁴⁹²/⁴⁹³, Ser⁵⁰¹, or Ser⁵⁰⁴. Reportedly, impaired internalization of the pd PTH1R, however, is not dependent on a specific phosphorylation site. In addition, we show that G protein-coupled receptor kinase 2 interferes with pd PTH1R signaling to Gs₁₁₁ proteins at least partially by direct binding to Gs₁₁₁ proteins.

G protein-coupled receptor kinases; phosphorylation

MATERIALS AND METHODS

Materials and plasmids. [Nle⁸,²¹,Tyr³⁴]rPTH(1-34)NH₂ (PTH, or PTH(1-34)) was kindly provided by A. Khatri (Endocrine Unit, Massachusetts General Hospital, Boston, MA). Chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The rat wt PTH1R cDNA cloned into pcDNA1, R15B (1), was used for single-strand plasmid preparation. Single or multiple alanine mutations were introduced by site-directed mutagenesis (24) at positions 489, 491, 492, 493, 495, 501, and 504 within the COOH-terminal tail of the PTH1R to produce the following receptor mutants: S489A, S501A and S504A, S491/492/493A, S493A, S492/493/495A, S489/491/492/493/495A, and S489/491/492/493/495/501/504A (pd PTH1R). These residues were phosphorylated upon stimulation with PTH, as shown previously (39). The mutations

Agonist-dependent activation of G protein-coupled receptors (GPCRs) is generally followed by initiation of a number of regulatory mechanisms leading to rapid signal attenuation, termed functional desensitization. Receptor phosphorylation by GPCR kinases (GRKs) and/or second messenger-dependent protein kinases (PKA or PKC) is regarded as an early step of functional desensitization. Other means of functional desensitization include rapid turnover of second messengers, β-arrestin binding, and receptor internalization.

Upon stimulation, the rat PTH1R becomes phosphorylated at seven serine residues within its COOH-terminal tail (34, 39). Similar findings were reported for the opossum PTH1R; however, only six serine residues were identified (27). Elimination of all seven phosphorylation sites, by serine-to-alanine mutations, results in enhanced and prolonged cAMP formation, as well as impaired internalization of the receptor when stably expressed in LLC-PK1 cells (39).

A mouse model in which a phosphorylation-deficient (pd) receptor was knocked into the locus of the PTH1R (pd PTH1R mouse) reiterates the above-described findings in vivo: subcutaneous injections of PTH led to enhanced cAMP secretion in the pd PTH1R mice compared with wild-type (wt) mice. Continuous infusions of PTH resulted in severe, progressive hypercalcemia in the pd PTH1R, but not wt, mice, which supports the hypothesis that receptor phosphorylation of the PTH1R negatively regulates PTH signaling (4).

We investigated whether activation of phospholipase C (PLC) by the pd PTH1R is altered and whether one or more substitutions of the serine residues identified as PTH-dependent phosphorylation sites of the PTH1R could reproduce the signaling abnormalities observed downstream of the pd PTH1R.

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were verified by sequencing. All mutants were examined for expression, and all have an expression level similar to or higher than that of the wt receptors. Plasmids of bovine GRK2, GRK3, GRK5, and GRK6 were kindly provided by Dr. J. L. Benovic (Thomas Jefferson University, Philadelphia, PA), and plasmids containing D110A-GRK2 or K220R-GRK2 were a gift from Dr. S. S. Ferguson (Robarts Research Institute, London, ON, Canada).

Cell culture and transfections. COS-7 cells were cultured in DMEM (Mediatech, Manassas, VA) supplemented with 10% FBS, 100 U/ml penicillin, and 1 μg/ml streptomycin at 37°C and 5% CO2. Cells were transiently transfected with different receptor constructs (2 μg total DNA/well) using diethylaminoethyl-dextran (Sigma-Aldrich) according to the manufacturer’s instructions. In all cotransfection experiments, the total amount of plasmid DNA was kept constant (2 μg/well) by addition of empty vector DNA or the second plasmid of interest at the desired ratio.

Cell surface receptor quantification. Quantification of cell surface receptors was performed as previously described (39). Briefly, COS-7 cells were plated onto 24-well plates 24 h after transfection and assayed 36–48 h later. After two rinses with Hanks’ balanced salt solution (Mediatech), cells were incubated with a receptor-specific antiserum (G48; 1:2,000 dilution; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to the cells for 90 min at room temperature. Cells were rinsed again before a 125I-labeled donkey anti-rabbit IgG F(ab’2) fragment (~250,000 cpm/well; GE Healthcare, Buckinghamshire, UK) was added for another 90 min at room temperature. Cells were then rinsed three times and solubilized with 1 N NaOH, and radioactivity was counted in a gamma counter (model 6400 Plus, Micromedic Systems, Horsham, PA). Receptor levels are expressed as percentage of total radioactivity bound (after subtraction of background radioactivity measured in vector-transfected COS-7 cells) normalized to basal or wt receptor levels depending on the experiment.

Inositol phosphate assays. Inositol phosphates were measured as previously described (41). Briefly, 48 h after transfection, the cells were incubated with 2 μCi/ml [myo-3H]inositol (18.6 Ci/mmol, Amersham, Buckinghamshire, UK) in serum- and myo-inositol-free DMEM for 12 h. Subsequently, the cells were stimulated with or without PTH in serum-free DMEM containing 20 mM HEPES (pH 7.4), 0.1% BSA, 100 mg/ml aprotinin, and 1 mM LiCl2. The stimulation was terminated by aspiration of the medium and addition of 3% perchloric acid. Intracellular inositol phosphates were separated by anion-exchange chromatography, and the radioactivity was determined by liquid scintillation counting in a liquid scintillation counter (Beckman Coulter, Fullerton, CA). Inositol phosphate values are expressed as the ratio of radioactivity incorporated into inositol phosphates to total radioactivity incorporated into both inositol phosphates and phosphatidyl inositols.

Data analysis. Inositol phosphate and receptor expression values calculated as described above were normalized to the control condition within each experimental setting and subsequently used for statistical analysis. All statistical/graphical analyses were done with Excel (Microsoft, Redmond, WA) or PRISM 3.02 (GraphPad, San Diego, CA). ANOVA followed by t-tests was utilized to compare experimental conditions using the different receptor/GRK constructs. Curves were fitted using classic equations for hyperbolic dose responses (PRISM 3.02). P < 0.05 was considered statistically significant.

RESULTS

PTH-dependent activation of the pd PTH1R leads to higher inositol phosphate accumulation. Upon PTH treatment, ligand binding and cAMP accumulation were not significantly different in COS-7 cells transfected with the wt or pd PTH1R (39) (data not shown).

In COS-7 cells, PTH1R stimulation leads to activation not only of adenylate cyclase but also, although less efficiently, of PLC. Since ligand and receptor conformations differentially affect downstream signaling pathways for the PTH1R (3, 6, 7, 37), we asked whether PLC stimulation was altered in cells expressing the pd PTH1R mutant under basal conditions or upon ligand stimulation. For this purpose, COS-7 cells were transiently transfected with the wt or pd PTH1R and assayed for inositol phosphate formation after PTH treatment. Initial time course experiments using 10−7 M PTH indicated that inositol phosphate accumulation was significantly increased in cells transfected with the pd PTH1R compared with the wt PTH1R, with the differences being more enhanced after 1 h than after 30 min of incubation (Fig. 1A). Therefore, 1-h incubations were chosen for all subsequent experiments. Upon stimulation with increasing concentrations of PTH, maximal
injected into COS-7 cells transiently transfected with wt PTH1R or pd PTH1R constructs. We previously demonstrated (39) that internalization of the pd PTH1R is significantly impaired compared with the wt PTH1R. Adjusted inositol phosphate formation in response to PTH treatment was significantly increased in cells transfected with the pd PTH1R, S491/492/493A, S501A, or S504A compared with those transfected with the wt PTH1R; these data likely reflect an intrinsic property of the pd receptors (Fig. 4).

Receptor internalization is impaired in COS-7 cells transiently expressing the pd PTH1R. We previously demonstrated that internalization of the pd PTH1R is significantly impaired in LLC-PK1 cells stably expressing this mutant receptor (39).
We therefore studied the internalization properties of the pd PTH1R in this transient expression system. In COS-7 cells transiently transfected with wt or pd PTH1R, receptor internalization reached a maximum 15–30 min after PTH addition and did not significantly change up to 1 h (data not shown). Therefore, we selected 1 h for comparison of inositol phosphate production and cell surface receptor levels. Receptor internalization after PTH treatment was significantly less for the pd PTH1R (all 7 serines substituted by alanines) than for the wt PTH1R after 30 min (data not shown) and 60 min (Fig. 5) of agonist incubation, confirming our previously published observations (39). However, none of the single or triple phosphorylation site mutants showed significantly altered internalization compared with the wt receptor (Fig. 5). We also tested a mutant receptor in which five serine residues were replaced by alanine residues (S489/491/492/493/495A), and again internalization upon PTH stimulation was not significantly different from the wt PTH1R when analyzed under the above-described conditions (Fig. 5). Collectively, our findings suggest that as few as one or two phosphorylated serine residues may be sufficient to mediate agonist-dependent internalization of the PTH1R.

GRK overexpression impairs inositol phosphate accumulation by interfering with pd PTH1R Gq/11 protein coupling. Formation of wt PTH1R-mediated inositol phosphate can be inhibited by GRK2, GRK3, and GRK5 overexpression (11). To obtain more mechanistic insights into how GRKs interfere with PTH-mediated PLC stimulation, we asked whether overexpression of the ubiquitously expressed GRK2, GRK3, GRK5, and GRK6 could interfere with increased inositol phosphate production through the pd PTH1R. We therefore expressed the wt or pd PTH1R together with GRK2, GRK3 and GRK5, or GRK6 (4:1 receptor-to-GRK ratio) and measured inositol phosphate formation in response to stimulation with 10−7 M PTH. Overexpression of GRK2, GRK3, GRK5, and GRK6 significantly reduced inositol phosphate production downstream of the wt or pd PTH1R. The magnitude of this effect on PLC stimulation did not differ for the wt or pd PTH1R (Fig. 6, for comparison of the wt and pd PTH1R, see reference line drawn at 50% inhibition of either control condition). This result suggests that all ubiquitously expressed GRKs interfere with PLC stimulation most likely independent of PTH1R phosphorylation and that receptor phosphorylation and functional desensitization by GRKs may be independent events at least with regard to PLC activation. Dicker and co-workers (11) demonstrated that both wt and a catalytically inactive GRK2 similarly inhibited PTH-mediated PLC stimulation through the PTH1R. Overexpression of the COOH terminus of GRK2 (aa 495-689) did not interfere with PTH-dependent PLC stimulation (11). The latter findings suggest that GRK2 may interfere with PLC activation through its relatively conserved NH2 terminus (33). The NH2 terminus of GRKs has been proposed to contain elements for Gq/11 protein-binding site by which they directly interact with Gq/11 proteins in an AlF4−-dependent manner (5). We therefore reasoned that the inhibitory effect of GRK2 overexpression on inositol phosphate production could be mediated by direct binding of GRK2 to Gq/11 proteins, as demonstrated for other GPCRs (8, 13, 20, 22, 26, 32). To test this hypothesis, we expressed either the wt or pd PTH1R together with wt GRK2 or mutant GRK2 constructs defective in Gq/11 protein binding (D110A) or catalytic activity (K220R) (36). In cells expressing the wt PTH1R, wt and mutant GRK2s (D110A and K220R) significantly inhibited PTH-dependent inositol phosphate production, suggesting that catalytic activity and direct binding of GRK2 to Gq/11 proteins were not necessary for GRK2 inhibition of PLC activation (Fig. 7). However, because of the low coupling efficiency of the wt receptor to Gq/11 protein (2- to 4-fold increase over basal inositol phosphate production with 10−7 M PTH in control condition), it is difficult to draw definitive conclusions from these experiments. Thus we coexpressed the pd PTH1R, which shows enhanced coupling to Gq/11 proteins, as shown earlier, with wt GRK2, K220R-GRK2, or D110A-GRK2. As demonstrated for the wt PTH1R, wt GRK2 and K220R-GRK2 significantly inhibited inositol phosphate formation in cells transfected with the pd PTH1R, suggesting that GRK2 effects on PTH-dependent PLC signaling are independent of receptor phosphorylation, as shown previously (11). However, overexpression of D110A-GRK2 reduced inositol phosphate formation to a significantly lesser extent than wt GRK2 (pd PTH1R + wt GRK2 vs. pd PTH1R + D110A-GRK2, P < 0.05; Fig. 7). These findings imply that inhibition of PTH-dependent inositol phosphate formation by GRK2 involves, in part, a direct interference of the kinase with Gq/11 proteins, at least for the pd PTH1R.

**DISCUSSION**

The present study explored the role of individual PTH1R phosphorylation sites in PTH1R signaling through PLC, agonist-dependent receptor internalization, and regulation by GRKs. We demonstrated by means of a heterologous expression system that PLC activation is dramatically enhanced downstream of the pd PTH1R and that this is not secondary to increased receptor numbers on the cell surface. It is conceivable, however, that impairment of agonist-dependent internalization may contribute to the dramatic enhancement of PLC stimulation of the pd PTH1R. However, internalization of the individual receptor mutants S491/492/493A, S501A, and
SS04A, which also showed enhanced PLC responses/receptors (Fig. 4), was not significantly different from wt receptors (Fig. 5). The latter findings suggest that increased inositol phosphate production mediated by these mutant PTH1Rs may reflect enhanced and/or prolonged coupling to G_{q/11} proteins. Disclosure of the exact molecular mechanisms for such a prolonged and enhanced coupling requires further investigation. The requirement for G_{q/11} proteins for the enhanced PLC stimulation by the pd PTH1R was recently established by the demonstration that small interfering RNA inhibition of G_{q/11} proteins, the pd PTH1R, as well as PTH1R mutants S491/492/493A, S501A, or S504A, confer significantly enhanced coupling to G_{q/11} proteins upon PTH stimulation. On the other hand, reportedly studies are necessary to dissect the mechanisms by which GRKs interfere with signaling of family B GPCRs.

In addition to enhanced signaling through G_{i} and G_{q/11} proteins, the pd PTH1R is significantly impaired in agonist-dependent internalization (39) (Fig. 5). Interestingly, this observation is only evident upon mutation of all seven serine residues to alanine residues. In addition, mutation of up to five phosphorylation sites did not negatively affect agonist-dependent internalization, suggesting that phosphorylation of any one or two serine residue(s) within the COOH-terminal tail of the PTH1R may be sufficient to promote agonist-stimulated receptor internalization. On the basis of the results, it is also possible that phosphorylation of Ser^{501} and Ser^{504} is necessary to mediate agonist-dependent receptor internalization.

Is there evidence that the distinct characteristics of pd PTH1R signaling contribute to regulation of mineral metabolism in vivo? Young male pd PTH1R mice are hypophosphatemic and hyperphosphaturic, pointing to enhanced and/or prolonged PTH1R actions within renal proximal tubules (4; S. U. Miedlich, unpublished observations). Regulation of renal phosphate handling by PTH is mediated by PKA- and PKC-dependent pathways (40). It is therefore possible that altered phosphate handling in the pd PTH1R mice may at least partly reflect enhanced PTH1R signaling via G_{q/11} and/or G_{i} protein-mediated pathways. Further studies with signaling-selective agonists and mouse models with selective PTH1R signaling (15, 17) are required to address whether and to what extent altered PLC signaling contributes to regulation of phosphate homeostasis by PTH. In fact, preliminary data (17) point to altered phosphate handling under conditions of chronic PTH stimulation (induced by calcium deficiency) in DSEL mice, which are selectively deficient in PLC but cAMP-dependent signaling of the PTH1R.

In conclusion, our work provides evidence that the pd PTH1R, as well as PTH1R mutants S491/492/493A, S501A, or S504A, confer significantly enhanced coupling to G_{q/11} proteins upon PTH stimulation. On the other hand, reportedly
impaired internalization of the pd PTH1R was not dependent on a specific phosphorylation site. In addition, we show here that GRK2, GRK3, GRK5, and GRK6 interfere with pd PTH1R signaling to Gq/11 proteins. The latter mechanism could potentially play a role as an adaptive mechanism in pd PTH1R-knockin mice, which outgrow the hypophosphatemic phenotype by 12 wk of age (S. U. Miedlich, unpublished observations).

ACKNOWLEDGMENTS

The authors thank Drs. Hesham A. Tawfeek, John T. Potts, Jr., Marie B. Demay, and Ernestina Schipani for continuous encouragement, critical discussions of the data, and careful review of the manuscript.

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AJP-Endocrinol Metab • VOL 295 • SEPTEMBER 2008 • www.ajpendo.org


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