Differential Conformational Requirements for Activation of G Proteins and the Regulatory Proteins Arrestin and G Protein-coupled Receptor Kinase in the G Protein-coupled Receptor for Parathyroid Hormone (PTH)/PTH-related Protein*

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After stimulation with agonist, G protein-coupled receptors (GPCRs) activate G proteins and become phosphorylated by G protein-coupled receptor kinases (GRKs), and most of them translocate cytosolic arrestin proteins to the cytoplasmic membrane. Agonist-activated GPCRs are specifically phosphorylated by GRKs and are targeted for endocytosis by arrestin proteins, suggesting a connection between GPCR conformational changes and interaction with GRKs and arrestins. Previously, we showed that by substitution of histidine for residues at the cytoplasmic side of helix 3 (H3) and helix 6 (H6) of the parathyroid hormone (PTH) receptor (PTHR), a zinc metal ion-binding site is engineered that prevents PTH-stimulated Gs activation (Sheikh, S. P., Vilardaga, J.-P., Baranski, T. J., Lichtarge, O., Iiri, T., Meng, E. C., Nissenson, R. A., and Bourne, H. R. (1999) J. Biol. Chem. 274, 17083–17091). These data suggest that relative movements between H3 and H6 are critical for Gs activation. Does this molecular event play a similar role in activation of GRK and arrestin and in PTH-mediated Gs activation? To answer this question, we utilized the two previously described mutant forms of PTHR, H401 and H402, which contain a naturally present histidine residue at position 301 in H3 and a second substituted histidine residue at positions 401 and 402 in H6, respectively. Both mutant receptors showed inhibition of PTH-stimulated inositol phosphate and cAMP generation in the presence of increasing concentrations of Zn(II). However, the mutants showed no Zn(II)-dependent impairment of phosphorylation by GRK-2. Likewise, the mutants were indistinguishable from wild-type PTHR in the ability to translocate β-arrestins/green fluorescent protein to the cell membrane and were also not affected by sensitivity to Zn(II). These results suggest that agonist-mediated phosphorylation and internalization of PTHR require conformational switches of the receptor distinct from the cAMP and inositol phosphate signaling state. Furthermore, PTHR sequestration does not appear to require G protein activation.

Transduction and regulation of signals by G protein-coupled receptors (GPCRs)1 require the interaction of the receptors with three families of proteins: G proteins, G protein-coupled receptor kinases (GRKs), and arrestins (1). Agonist binding to GPCRs allows G proteins to bind to the cytoplasmic parts of the receptor and to become activated by accelerating GDP/GTP exchange of the G protein α-subunits of the heterotrimer (2). This event promotes activation of effector enzymes and ion channels by the activated Go-GTP as well as the Gβγ complex (3). Signaling by most agonist-activated GPCRs is regulated by a two-step process (also referred to as the rapid form of GPCR desensitization) in which the activated receptors become a target for phosphorylation by GRKs and then interact with cytosolic arrestin proteins (1, 4). Binding of arrestin to the agonist-activated receptor has at least two consequences: uncoupling of receptors from G proteins, resulting in termination of agonist-mediated signaling, and facilitation of receptor internalization (5, 6).

A common feature of G proteins, GRKs, and arrestins is their specific binding of and activation by agonist-occupied receptors. This suggests a link between the receptor conformational changes and the binding of these proteins by the receptor. Is the receptor conformational switch the same for activation of G proteins, GRKs, and arrestins? Studies based on constitutively active α2- and β2-adrenergic receptors (7, 8) have shown that these receptors are also constitutively phosphorylated and desensitized. This supports the model that the active state of the receptor is identical to the conformation required for phosphorylation and internalization. In other words, agonist binding to GPCR induces an intramolecular rearrangement of the receptor domains (transmembrane helices and intracellular loops), resulting in a generalized receptor conformation able to promote activation of G proteins, phosphorylation by GRKs, and binding of arrestins. In contrast, two recent studies (9, 10) demonstrated that constitutively active angiotensin type 1 and α1B-adrenergic receptors resulting in agonist-independent signal did not show an increase in basal phosphorylation. Thus, constitutively active receptors may have contrary effects on coupling to G proteins and GRKs. These studies leave open the question concerning the existence of one or multiple states of the activated receptor to promote activation of G proteins and to bind regulatory proteins.

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1 The abbreviations used are: GPCRs, G protein-coupled receptors; GRK, G protein-coupled receptor kinase; PTH, parathyroid hormone; PTHR, parathyroid hormone receptor; GFP, green fluorescent protein; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; GTPγS, guanosine 5’-O-(3-thiotriphosphate); IP, inositol phosphate.
Recently, nitroxide spin-spin interaction experiments demonstrated that the molecular nature of the conformational change in rhodopsin associated with light-induced transducin (G<sub>α</sub>) activation is linked to a relative movement and rotation of the cytoplasmic part of helix 3 away from the cytoplasmic part of helix 6 (11). In support of this model, it was demonstrated that by engineering an interhelical zinc ion bridge to constrain the movements between helices 3 and 6 in rhodopsin, G<sub>α</sub> activation is inhibited (12). Using a similar zinc ion site engineering strategy, we have shown the importance of the relative movement between helices 3 and 6 for G<sub>α</sub> activation in the β<sub>2</sub>-adrenergic receptor and the parathyroid hormone receptor (PTHr) (13). Altogether, these studies indicate that the relative motion between helices 3 and 6 is a common switch mechanism in GPCRs of class I (rhodopsin-like) and class II (PTHr-like) to activate G proteins. A central question remains of whether the agonist-induced movement of helices 3 and 6 also acts as a molecular switch to control activation of different G protein subtypes coupled to the same receptor and of other agonist-activated GPCR regulatory proteins such as GRKs and arrestins.

After stimulation by PTH, PTHr assumes an active state that activates G<sub>α</sub> and G<sub>β</sub>γ proteins to stimulate adenylyl cyclase and G<sub>q</sub> proteins to stimulate phospholipase C, respectively (14). Agonist-induced activation of PTHr results in its phosphorylation by GRK-2 (15, 16) and in the rapid internalization of the PTH-receptor complex via an arrestin-dependent pathway (17). Is the same G<sub>α</sub> protein-receptor active state required for PTH-stimulated activation of G<sub>α</sub>, GRK, and arrestin? To test this hypothesis, we employed two mutants of PTHr that we have previously described, H401 and H402 (13). These mutants carry a naturally present His residue at position 301 in helix 3 (near the junction of the second intracellular loop) and a His residue substituted for Leu<sup>401</sup> (mutant receptor H401) or Lys<sup>402</sup> (mutant receptor H402) in helix 6 (near the junction of the third intracellular loop). We demonstrated previously that in the presence of Zn(II), receptors H401 and H402 were able to form an artificial Zn(II) bridge between helices 3 and 6 of the receptor that inhibits PTH-mediated activation of G<sub>α</sub> (13). Our present data indicate that the conformational change leading to relative movement of helices 3 and 6 is also required for G<sub>α</sub> activation, but is not involved in the activation process of the regulatory proteins GRK-2 and arrestin. This implies that there are different conformational requirements for G protein activation and for coupling to GRKs and arrestins and that free movement of helices 3 and 6 is specifically necessary for G protein activation. Taking advantage of the fact that in the presence of Zn(II), H401 and H402 signaling is blocked, we have directly examined the role of G protein activation in PTHr internalization.

**EXPERIMENTAL PROCEDURES**

**Materials**—PTH-(1–34) was obtained from Bachem, and ZnCl<sub>2</sub> was from Sigma. The generation of PTHr mutants H401 and H402 has been described previously (13). β-Arrestin-1 was fused to green fluorescent protein (GFP) as described (18). The cDNA for β-arrestin-2 was a generous gift from Dr. S. Cotechia (Institut de Pharmacologie et de Toxicologie, Université de Lausanne). The stop codon of this cDNA was replaced with an XbaI restriction site and fused to the cDNA of GFP (pGreenLantern I, Life Technologies, Inc.). Radiolabeled PTH-(1–34) (2200 Ci/mmol) was kindly provided by Dr. Eberhard Blind (Department of Endocrinology, University of Würzburg). The anti-β-arrestin antibody has been described previously (19). During this study, it was shown that this antibody recognizes β-arrestin-1 only, but not β-arrestin-2.

**Receptor Expression**—COS-7 cells (American Type Culture Collection) served as the expression system for the wild-type and mutant receptors. Cells were maintained in culture at 37 °C under a humidified atmosphere with 7% CO<sub>2</sub> in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Life Technologies, Inc.). Transient transfections were performed using a protocol based on the DEAE-dextran method as described previously (20). For confocal scanning microscopy, HEK-293 cells were used; cells were grown on coverslips previously coated with poly-L-lysine and transfected by the phosphate precipitation method (20).

**Membrane Preparation**—Cells were harvested, lysed in buffer A (20 mM Tris, pH 7.4, 2 μg/ml aprotinin, 10 μg/ml benzamidine, and 100 μg/ml Pefabloc), and homogenized. After centrifugation for 20 min at 40,000 × g, the pellet was resuspended in buffer A with 20% sucrose and layered onto 40% sucrose in buffer A. After centrifugation at 125,000 × g for 1 h, the 20/40% interface was recovered, diluted 1 + 1 with buffer A, and centrifuged for 15 min at 125,000 × g. The pellet was resuspended in 5 mM urea, incubated for 10 min at 20 °C, and centrifuged at 125,000 × g for 15 min. After two washing steps in buffer A, the final pellet was resuspended in the same buffer and stored at −80 °C.

**Expression and Purification of GRK-2—GRK-2 was purified from SF9 insect cells as described previously (21) and stored in 20 mM Hepes, 5 mM EDTA, pH 7.2, 100 mM NaCl, and 0.1 mM dodecyl maltoside at 4 °C. GRK-2-specific activity (0.98 ± 0.30 mU of PThr/min/mg of kinase) was determined with rhodopsin as the substrate and 10 mM kinase, 2.4 μM rhodopsin, and 100 μM ATP.

**Membrane Phosphorylation Assays**—In the following assays (e.g. phosphorylation, ligand binding, cAMP, inositol phosphate, internalization, and arrestin translocation), to allow zinc ion to form a complex with the H401 or H402 receptor, intact cells or membrane preparations containing receptors were incubated with or without concentrations of ZnCl<sub>2</sub> in the indicated buffer for 10–15 min before the start of the assay. Receptor-containing cell membranes (10 μg of total proteins) were incubated with purified GRK-2 (100 nm) in 20 mM Tris, pH 7.4, and 8 mM MgCl<sub>2</sub> containing 100 μM [γ<sup>32</sup>P]ATP (1–2 cpm/fmol) and the indicated concentrations of ZnCl<sub>2</sub> in a final volume of 25 μL. PTH-(1–34) (10–1000 nm) was added as indicated, and the incubations were carried out for 30 min at 30 °C. Reactions were stopped by addition of Laemmli buffer, followed by electrophoresis on SDS-polyacrylamide gels. [32P]-Labeled proteins were visualized and quantified by Phosphorimage analysis.

**Determination of Receptor Internalization**—Transfected cells were grown for 48 h in six-well dishes. After incubation for 1 h at 37 °C with 1 mL of DHB buffer (serum-free Dulbecco’s modified Eagle’s medium containing 20 mM Hepes and 1% bovine serum albumin), the cells were incubated at 37 °C for the indicated times with 1 mL of DHB buffer containing [125I]-PTH-(1–34) (100,000 cpm) and the indicated concentrations of ZnCl<sub>2</sub>. Incubations were stopped by washing the cells on ice and rapidly washing them with 1 mL of ice-cold phosphate-buffered saline. Cells were then incubated for 2 × 5 min in 0.5 mL of acid wash solution (150 mM glycine and 50 mM acetic acid, pH 3) to remove the surface-bound radioactivity. The supernatants containing the acid-released radioactivity were collected, and the cells were treated with 1 mL of 0.8 N NaOH to solubilize the acid-resistant radioactivity. Non-specific binding was measured in parallel experiments using 10–6 M PTH-(1–34). Radioactivity was quantified in a γ-counter. The percent internalization was calculated after deduction of the respective non-specific value: % internalization = (cpm acid-resistant/cpm acid-resistant × 100) × 100.

**Assays for Receptor Function**—Ligand binding, cAMP, and inositol phosphate assays were carried out following previously published procedures (15, 16). In brief, for binding studies in intact cells, transfected cells were incubated in DHB buffer for 2 h at 0 °C, followed by a 2-h incubation with the same buffer containing [125I]-PTH-(1–34) (100,000 cpm) with or without unlabeled PTH-(1–34). Cells were washed twice with ice-cold phosphate-buffered saline and extracted with 0.8 N NaOH, and cell-associated [125I]-PTH-(1–34) was counted. Competition curves were fitted to a one-site competitive binding curve. [125I]-PTH-(1–34) binding experiments with membranes (50 μg) were done in 100 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 50 mM Tris, pH 7.4, 0.1% CHAPS, 1% bovine serum albumin, and 100 μg of bacitracin. After incubation for 90 min at 22 °C in the absence or presence of GTPγS and/or ZnCl<sub>2</sub>, bound radioactivity was separated from free radioactivity by centrifugation at 20,000 × g for 10 min at 4 °C. The pellets were washed three times with ice-cold binding buffer and counted. Non-specific binding was defined using 1 μM PTH-(1–34).

For inositol phosphate assays, transfected cells in six-well plates were incubated for 1 h at 37 °C with 1 μCi/ml myo-[2-3H]inositol in Dulbecco’s modified Eagle’s medium without inositol. After labeling, cells were washed twice with Hepes buffer (137 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 20 mM Hepes, pH 7.3) and incubated in 900

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μl/well of the same buffer with 10 mM LiCl for 10 min. Total inositol phosphate accumulation was measured after 1 h of stimulation at 37 °C by 1 μM PTH-(1–34) with or without the indicated concentrations of ZnCl₂. Extraction and determination of total inositol phosphates were done as described (15). Inositol phosphate responses were expressed as percent of the control response at 10⁻⁶ M PTH-(1–34) after subtracting the respective basal levels present in unstimulated cells. For cAMP assays, cells were washed twice with Hepes buffer and incubated with isobutylmethylxanthine (0.5 mM) in the same buffer for 5 min at room temperature. Cells were stimulated with 100 nM PTH-(1–34) in the absence or presence of different concentrations of ZnCl₂ at 37 °C for 10 min. Cellular cAMP was extracted and measured by radioimmunoassay (Immunotech).

Assays for β-Arrestin Translocation—β-Arrestin translocation was monitored by two assays: confocal scanning microscopy visualization in intact cells and immunoblot analysis. HEK-293 cells cotransfected with β-arrestin-2 fused to GFP and PTHr cDNAs were incubated with 100 nM PTH-(1–34) for different times and fixed in 4% paraformaldehyde. Fixed cells were observed under an oil immersion objective (×40) using a Leica TCS confocal laser microscope. Representative optical sections corresponding to the middle of the cells are presented. For immunodetection, solubilized membrane proteins and soluble protein fractions of transfected cells coexpressing PTHR and β-arrestin-1/GFP were separated on SDS-10% polyacrylamide gel, and proteins were transferred onto an Immobilon P transfer membrane (Millipore Corp., Bedford, MA). Membranes were reacted with anti-β-arrestin-1 antibody (1:3000), and immunoreactive bands were visualized by anti-rabbit Ig (1:20000) coupled to peroxidase and chemiluminescence (Pierce).

Data Analysis—Concentration-response, competition, and internalization kinetics curves were analyzed by computer-assisted nonlinear regression (GraphPAD Software for Science, San Diego, CA). If not otherwise indicated, all experiments were replicated at least three times in independent experiments, and the results are expressed as the means ± S.E.

RESULTS

PTH Binding Affinities and Signaling Characteristics for PTHR, H401, and H402—Competition of PTH for ¹²⁵I-PTH binding on intact cells gave similar Kᵢ values for wild-type and mutant receptors (Fig. 1, A and B): PTHR, Kᵢ = 4.5 ± 0.4 nM (n = 10); H401, Kᵢ = 9.5 ± 1.0 nM (n = 4); data not shown); and H402, Kᵢ = 10.1 ± 0.9 nM (n = 4). In the presence of Zn(II) (100 μM), a small increase in PTH binding affinity was observed for H401 (Kᵢ = 5.3 ± 0.6 nM, n = 4) and H402 (Kᵢ = 7.1 ± 0.9 nM, n = 4). The absence of a decrease in PTH binding affinity suggests that Zn(II) did not affect G protein coupling to the receptors. On cell membrane preparations, the presence of 20 μM GTPγS reduced the ¹²⁵I-PTH-specific binding by ∼42% for PTHR and by ∼40% for H402 (Fig. 1C). 100 μM Zn(II) failed to reduce the specific tracer binding on both receptors, and addition of GTPγS caused a similar decrease in tracer binding of both receptors (experiments not done for H401). The failure of Zn(II) to reproduce the effect of GTPγS on tracer binding confirms that Zn(II) did not affect the G protein coupling to both receptors. Fig. 1D summarizes PTH-induced PTHR and H402...
signaling on intact cells (cAMP and inositol phosphate turnover) and phosphorylation by GRK-2 in membrane preparations. The concentrations required for 50% of the maximal effect were moderately but significantly increased for the H401 and H402 mutants: for cAMP, PTHR = 1.2 ± 0.4 nM (n = 7), H401 = 5.2 ± 0.8 nM (n = 3), and H402 = 8.6 ± 0.7 nM (n = 3); for inositol phosphate turnover, PTHR = 100 ± 9 nM (n = 5), H401 = 210 ± 12 nM (n = 3), and H402 = 225 ± 35 nM (n = 3); and for phosphorylation, PTHR = 9.1 ± 0.8 nM (n = 5) and H402 = 7.5 ± 1.5 nM (n = 3) (experiments not done for H401).

Receptor Molar Switch for Gq-mediated Signaling Is Similar to That Required for Gs Activation—To assess activation of Gs by wild-type and mutant PTHRs, we measured PTH-mediated inositol phosphate (IP) turnover in intact cells. Wild-type PTHR and mutants H401 and H402 did not show increased basal IP signaling activity in the presence of 1–1000 μM Zn(II). As shown in Fig. 2A, a high concentration of Zn(II) inhibited the ability of activated wild-type PTHR to stimulate IP synthesis (IC50 = 78.5 ± 5.5 μM, n = 5). The sensitivity of PTH-stimulated IP synthesis to Zn(II) was significantly increased in cells expressing H401 (IC50 = 14.9 ± 1.8 μM, n = 4) or H402 (IC50 = 3.3 ± 0.5 μM, n = 3). In our previous study (13), similar curve shifts were observed for PTH-stimulated GTPγS binding to Goα, in membrane preparations (Fig. 2B). Altogether, these data indicate that zinc ion occupancy of the engineered site within H401 and H402 blocks Gs activation as well as the Gq-mediated IP signal in response to PTH. A relative movement of the two helices is the conformational change that best accounts for the inhibitory effect of Zn(II) on H401 and H402 signaling. Thus, a relative movement between helices 3 and 6 in PTHR is required for activation of Gs-mediated IP synthesis.

The occurrence of a nonspecific increase in basal cAMP in cells or of basal activity of adenylyl cyclase in membrane preparations and inhibition (~40%) of forskolin-mediated adenylyl cyclase stimulation by ZnCl2 (10–1000 μM) made it difficult to quantify the effect of Zn(II) on the PTH-mediated cAMP response of wild-type and mutant receptors (data not shown). Despite these impediments, Fig. 2C highlights a differential effect of Zn(II) between PTHR and H402 on cAMP accumulation. Indeed, the Zn(II) capability to inhibit 1 μM PTH-stimulated cAMP synthesis was higher for H402 than for PTHR (IC50 = 256 ± 35 versus 1300 ± 150 μM, n = 3). However, the Zn(II) effect on cAMP was smaller than on IP turnover (IC50 decrease of ~34-fold for IP versus ~5-fold for cAMP). In fact, Zn(II) sensitivity was more effective at 10 nM PTH-mediated cAMP synthesis (IC50 = 7.5 ± 1.2 μM (n = 2) for H402 versus IC50 = 193 ± 20 μM (n = 2) for PTHR). A possible explanation for such differences in the Zn(II) effect on PTH-stimulated cAMP and IP turnover might be due to the more efficient coupling of PTHR to the adenylyl cyclase machinery (see “Discussion”).

High doses of PTH (1 μM) were required for maximal stimulation of IP turnover, whereas ~100-fold less PTH was necessary for maximal cAMP stimulation (Fig. 1, D and E).

Molecular Switches Required for Receptor Phosphorylation by GRK Are Distinct from That Necessary for G Protein Activation—We previously demonstrated that PTHR is efficiently phosphorylated by GRK-2 both in cell membrane preparations and in intact cells (15, 16). GRK-2-mediated receptor phosphorylation was measured in cell membrane preparations containing wild-type or H402 mutant PTHR. Fig. 3 shows that the H402 receptor was recognized by the kinase GRK-2, resulting in substantial PTH-dependent phosphorylation similar to that observed for wild-type PTHR. Wild-type and H402 receptors displayed a basal level of phosphorylation that was efficiently stimulated by PTH (~3-fold increase over the basal level). The presence of ZnCl2 (1–1000 μM) did not influence the basal level of receptor phosphorylation (data not shown). 10 μM PTH-mediated phosphorylation of both PTHR and the H402 receptor was not modified by 1–100 μM Zn(II) (Fig. 3A). High concentrations of ZnCl2 (1 mM) have previously been shown to inhibit GRK-2 (23) and indeed inhibited by 50% the ability of GRK-2 to phosphorylate both receptors in response to PTH (Fig. 3A). The presence of 100 μM Zn(II) (a concentration that blocks activated H402-mediated cAMP and IP synthesis in intact cells (Fig. 2, A and C) as well as Gs activation in membrane preparations (13)) did not modify the capability of lower concentrations of GRK-2 to phosphorylate PTHR (data not shown) or H402 (Fig. 3B). The above data indicate that Zn(II) complexed to the H402 receptor is able to form a conformation in response to PTH that results in GRK-2-mediated phosphorylation (but not activation) of Gs and Gq proteins. These data demonstrate that the
signaling state of the receptor can be differentiated from the phosphorylatable state by blocking a motion between helices 3 and 6 of the receptor.

**Motion between Helices 3 and 6 of PTHR Is Dispensable for β-Arrestin-2-mediated Receptor Internalization**—Although agonist-induced phosphorylation of GPCRs is an important determinant for arrestin interaction with the receptors, we recently found that the GRK phosphorylation site in PTHR is not critical for β-arrestin interaction with the receptor. Thus, the receptor conformational changes appear to be crucial for arrestin interaction. We next considered whether β-arrestin-mediated internalization of the receptor is dependent on the relative movement between helices 3 and 6. The ability of PTH to stimulate wild-type and mutant PTHR internalization was monitored by following the internalization kinetics of $^{125}$I-PTH. Fig. 4A shows that the internalization of PTHR was rapid and efficient ($k_0 = 0.15 \pm 0.05$ min$^{-1}$; maximum = 60%; $n = 7$) and similar to the internalization of H401 ($k_0 = 0.20 \pm 0.05$ min$^{-1}$; maximum = 59.7%; $n = 5$) and H402 ($k_0 = 0.17 \pm 0.03$ min$^{-1}$; maximum = 55%; $n = 4$). The internalization properties of PTHR, H401, and H402 were not influenced by Zn(II) (1 μM) (data not shown). In the presence of 1 mM Zn(II), a small increase in the endocytic rate constant was observed for all the receptors: PTHR, $k_0 = 0.25 \pm 0.03$ min$^{-1}$; H401, $k_0 = 0.20 \pm 0.05$ min$^{-1}$; and H402, $k_0 = 0.20 \pm 0.08$ min$^{-1}$. Overexpression of β-arrestin-2 significantly promoted PTH-mediated internalization of PTHR ($k_0 = 0.20 \pm 0.07$ min$^{-1}$; maximum = 70.9%; $n = 5$) and H402 ($k_0 = 0.23 \pm 0.06$ min$^{-1}$; maximum = 69.3%; $n = 3$) (Fig. 5A) (data not shown for H401). Zn(II) (1–100 μM) did not affect the ability of β-arrestin-2 to enhance sequestration of both PTHR and the H402 receptor (Fig. 5B) (data not shown for H401). From these data, we assume that in response to PTH, β-arrestin-2 was able to promote internalization of the conformationally constrained H401 and H402 receptors. Thus, β-arrestin-2 was not sensitive to restriction in the movement between helices 3 and 6 of the receptor.

To examine more directly the interaction of arrestins with PTHR, we evaluated PTH-induced translocation of β-arrestin-2/GFP in HEK-293 cells (Fig. 6, A and B). β-Arrestin-2/GFP and β-arrestin-2 caused an identical improvement of PTH-induced PTHR internalization (data not shown). As PTHR functions (signaling and sequestration) are similar in HEK-293 and COS cells, HEK-293 cells were preferred to COS cells for immunofluorescence assays because their shape makes them more suitable for visualization of the translocation. After 5–10 min of PTH stimulation, an efficient redistribution of β-arrestin-2/GFP from the cytosol to the cell membrane was observed in cells expressing wild-type PTHR. After 20 min in the presence of PTH, β-arrestin-2/GFP clusters appeared on the cell membrane periphery. Similar results were seen with cells expressing the H401 or H402 receptor (Fig. 6B) (data not shown for H401). Indistinguishable β-arrestin-2/GFP translocations were observed with cells exposed to different concentrations of Zn(II) (10–1000 μM) (Fig. 6, A and B) (data not shown for 10 μM Zn(II)). This indicates that the ability of PTH-occupied receptors to translocate arrestins to the periphery of the cells is not dependent on the motion between helices 3 and 6 of the receptor.

Furthermore, we quantified arrestin translocation by analysis of cellular localization of β-arrestin-1/GFP by Western blotting of COS cell lysates. β-Arrestin-1/GFP and β-arrestin-2/GFP displayed similar membrane translocation in response to PTH. In unstimulated cells coexpressing β-arrestin-1/GFP and PTHR or H402, no β-arrestin-1/GFP was detected in the membrane fraction (Fig. 7A). After 5 and 10 min of PTH (100 nM) exposure, we observed a considerable β-arrestin-1/GFP translocation to the cell membrane containing PTHR or H402 (Fig. 7B). No translocation in response to PTH was observed in cells transfected only with β-arrestin-1/GFP (data not shown). At later times (20 min), β-arrestin-1/GFP associated with membrane diminished by 15–20% for cells expressing PTHR or H402 (Fig. 7B). No significant difference in the profile of time-dependent β-arrestin-1/GFP translocation was seen in cells expressing PTHR or H402 and treated with 100–1000 μM Zn(II) (Fig. 7B). From these data, we infer that a conformational constraint of the receptor (H402 in the presence of Zn(II)) does not block arrestin binding to the receptor or the capability of arrestins to promote receptor internalization.

**DISCUSSION**

GPCRs are membrane molecular switches that activate three families of proteins, G proteins, GRKs, and arrestins, in response to a large variety of extracellular stimuli (light, odors, small molecules, peptides, and proteins). Relative movement and/or translation among the seven GPCR transmem-

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2 J.-P. Vilardaga, C. Krasel, S. Chauvin, T. Bambino, R. A. Nissenson, and M. J. Lohse, manuscript in preparation.

3 J.-P. Vilardaga, R. A. Nissenson, and M. J. Lohse, unpublished observations.
brane helices is thought to mediate receptor activation. Presently, little is known about the GPCR conformational changes that promote activation of proteins coupled to receptors such as G proteins, GRKs, and arrestins. In a previous study, we used membrane preparations from COS cells expressing receptors containing an engineered zinc ion bridge between helices 3 and 6 of PTHR to successfully inhibit, by the tracer binding suggest that 

Unique Receptor Conformational Change Is Required to Activate Multiple G Protein Subtypes—It was possible to verify in intact cells whether movement between helices 3 and 6 is associated with activation of different G proteins. Indeed, Zn(II) (~100 μM) selectively blocked the ability of H401 and H402 to mediate G_s/phospholipase C signaling. Because adding GTPγS (a GTP analog) causes the dissociation of G protein subunits (α and βγ) from each other and from the receptor (24), the decrease in [125I]-PTH binding observed in the presence of GTPγS is thought to reflect the dissociation of PTHR from cognate G proteins. The lack of a decrease in PTH binding affinity in the presence of Zn(II) and the failure of Zn(II) to mimic the effect of GTPγS on the tracer binding suggest that Zn(II) did not disrupt G protein binding to the receptor. Thus, as previously proposed in our earlier study (13), the ability of a Zn(II) bridge to block H401 and H402 signaling resides in its ability to inhibit G protein activation rather than G protein coupling. Compared with the strong Zn(II) sensitivity of H402 in inhibiting activation of G_s in vitro and of the 1 μM PTH-mediated G_s/phospholipase C pathway in intact cells, we observed an apparently weaker effect of Zn(II) in blocking the 1 μM PTH-mediated G_q/adenyl cyclase pathway. Adenyl cyclase inhibition by Zn(II) was more effective when the receptors were stimulated with 10 rather than 1000 nM PTH (Fig. 2C). The differences in Zn(II) sensitivity between 1 μM PTH-mediated cAMP and IP synthesis might be related to the relative affinity of G_q and G_s for the receptors. Indeed, Fig. 1 (D and E) shows that the coupling of G_q to the receptors is more efficient than of G_s, suggesting a higher affinity of G_q for the receptors. Since the Zn(II) apparent affinity for H402 is low (i.e. micromolar range), G_s proteins with a high affinity for the receptor would then be less susceptible to the Zn(II) effect. The Zn(II) effect on PTH-mediated cAMP production should be more pronounced if low concentrations of receptor are activated, which is exactly what was observed (Fig. 2C).

Our findings suggest that the relative movement of helices 3 and 6 of the receptor is required for activation of both G_q and G_s protein subtypes by PTHR.

How Do GRK and Arrestin Respond to Activated GPCR When the Motion between Helices 3 and 6 Is Blocked?—Based on the facts that constitutively active receptors display enhanced agonist-independent phosphorylation (7, 8) and that partial agonists cause partial receptor phosphorylation (25), it was inferred that the receptor signaling state is also the conformation...
for GRK-mediated phosphorylation. In support of this conclusion, studies based on rhodopsin have demonstrated that the light-activated rhodopsin conformation, metarhodopsin II, can activate transducin (Gt protein) and is the substrate for rhodopsin kinase (GRK-1) (1, 4), supporting the model that the metarhodopsin II state has the conformation required for activation of Gt and GRK-1. In contrast, recent studies demonstrated that the constitutively active \( \beta H1\-H9251\) adrenergic receptor (mutations D142A and D142T) (10) and angiotensin type 1 receptor (mutation N111A) (9) show no increase in basal phosphorylation, nor is their phosphorylation stimulated by agonist. This suggests that multiple receptor states might exist for receptor activation and that not all are a target for phosphorylation. If the conformational switch for activation of G proteins and GRK is similar, we would expect, in the presence of Zn(II), a decrease in the ability of GRK-2 to phosphorylate the PTH-occupied H402 receptor. This was not observed. Indeed, despite the inability of the H402 receptor in the presence of Zn(II) to promote Gs activation or \( G_\alpha\)-mediated IP signaling, phosphorylation of H402 by GRK-2 in the presence of Zn(II) was as efficient as that observed for wild-type PTHR. Therefore, our data seem consistent with a model in which the receptor conformation necessary for G protein activation is different from the one required for GRK-mediated receptor phosphorylation.

A similar distinction can be drawn between activation of G proteins and arrestins. \( \beta\)- Arrestins discriminate between the inactive and active conformation of most GPCRs (5, 26, 28, 29) and act as adaptors, binding both activated receptor and clathrin to promote agonist-occupied receptor sequestration via a clathrin-coated pit pathway (27). The current model for arrestin activation proposes that arrestin recognizes two binding sites on GPCRs: an activation site that is exposed upon receptor activation and a GRK-phosphorylated site (28–31). Importantly, however, we recently observed that a phosphorylation-deficient PTHR is capable of binding \( \beta\)-arrestin-1 and \( \beta\)-arrestin-2 efficiently in response to PTH (32).2 This suggests that the GRK-phosphorylated site in GPCRs is not always critical for firm arrestin interaction with PTHR. Rather, the activation recognition site appears to be crucial for arrestin binding. Recent studies on rhodopsin have indicated that transducin and arrestin compete for overlapping interaction sites on metarhodopsin II (33), suggesting that G protein and arrestin recognize the same active receptor conformation. The fact that Zn(II) occupancy in the H402 receptor impaired its ability to cause PTH-induced Gs and Gt activation, but not its ability to mobilize arrestin efficiently, indicates that the conformational changes necessary to activate arrestin or G protein are different.
Is G Protein Activation Required for Receptor Internalization?—It is unclear whether the same active conformation of GPCRs is required for activation of G proteins and for receptor sequestration. Indeed, conclusions from experiments using partial agonists and signaling-impaired receptors have been contradictory. Studies suggesting that GPCRs internalize as a consequence of G protein activation are based on two kinds of facts: first, a linear correlation between agonist activity and rate of receptor endocytosis has been proposed for the m3 muscarinic receptor (34), the lutropin/choriogonadotropin receptor (35), and the β2-adrenergic receptor (36); second, point mutations that impair signal transduction of the lutropin/choriogonadotropin receptor (37), the β2-adrenergic receptor (38), the thyrotropin-releasing hormone receptor (39), and PTHR (32) also impair their agonist-mediated receptor internalization. In contrast, other studies have reached an opposite conclusion. Indeed, point mutations of the β2-adrenergic receptor (40) and the angiotensin type 1 receptor (41) have been described that impair receptor signaling, but do not suppress receptor internalization. The fact that 100 and 1000 μM Zn(II) selectively blocked H402 signaling via both Gq and Gs allowed us to examine more directly the role of G protein activation in PTH-induced receptor internalization. We observed a failure of Zn(II) (100–1000 μM) to block PTH-induced receptor internalization, β-arrestin translocation, or the ability of β-arrestin to enhance receptor sequestration. Thus, selective inhibition of PTH-mediated G protein activation had no consequence on PTHR internalization via the arrestin pathway. Our findings suggest that G protein activation and receptor internalization are controlled by distinct agonist-induced conformational switches of the receptor.

Using inhibitors (staurosporine) and activators (phorbol 12-myristate 13-acetate) of protein kinase C, it was previously reported that PTHR internalization requires protein kinase C activation (17). Our results, which show that the Gq/phospholipase C pathway could be blocked with no outcome on receptor internalization and β-arrestin translocation, make it improbable that PTHR-mediated activation of protein kinase C is an obligatory requisite for receptor endocytosis via an arrestin pathway. In conclusion, we demonstrated that the agonist-mediated relative movement of helices 3 and 6 of PTHR known to be involved in Gq protein activation is also true for the activation process of Gs protein. However, the separation between helices 3 and 6 is not involved in GRK activation or arrestin-mediated receptor endocytosis. Our findings suggest the existence of two separate conformational switches in the receptor (e.g., conformations A and B) that can differentially activate G proteins (receptor conformation A) and GRK and arrestin (receptor conformation B). To integrate the fact that GRKs and arrestin compete for interaction sites on activated rhodopsin, another possible interpretation is that G protein couples to receptor conformation A and that GRK and arrestin recognize both receptor conformations A and B. In this case, Zn(II) would prevent formation of receptor conformation A, but not of receptor conformation B. It remains to be determined what kinds of agonist-induced interhelical conformational changes within the receptor are required for activation of arrestin and GRKs.

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Differential Conformational Requirements for Activation of G Proteins and the Regulatory Proteins Arrestin and G Protein-coupled Receptor Kinase in the G Protein-coupled Receptor for Parathyroid Hormone (PTH)/PTH-related Protein
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