Selective Ligand-induced Stabilization of Active and Desensitized Parathyroid Hormone Type 1 Receptor Conformations*

Received for publication, March 15, 2002, and revised form, June 20, 2002
Published, JBC Papers in Press, July 9, 2002, DOI 10.1074/jbc.M202544200

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For many G protein-coupled receptors, agonist-induced activation is followed by desensitization, internalization, and resensitization. In most cases, these processes are dependent upon interaction of agonist-occupied receptor with cytoplasmic β-arrestins. The ligand-induced intramolecular rearrangements of the receptor responsible for the observed activation of effector systems, which dictate both the pharmacological properties of ligands and the biological activity of G protein-coupled receptors, have not been fully elucidated. Here, we identify specific interactions between parathyroid hormone (PTH)-related protein and the human PTH type 1 receptor (PTH1Rc) and the related receptor conformational changes that lead to β-arrestin-2-mediated desensitization. PTH-related protein analogs modified at position 1 induced selective stabilization of the active G protein-coupled state of the receptor, resulting in lack of β-arrestin-2 recruitment to the cell membrane, sustained cAMP signaling, and absence of ligand-receptor complex internalization. Mechanistically, the ligands modified at position 1, interacting with the extracellular end of helix VI of PTH1Rc, produced a translocation of transmembrane helices V and VI that differed from that induced by the cognate agonist, resulting in significantly different conformations of the third intracellular loop. These results show that the extracellular interactions between PTH1Rc and its ligands may stabilize distinct conformational states, representing either the active G protein-coupled or a desensitized β-arrestin-coupled receptor state. In addition, they establish that sustained biological activity of PTH1Rc may be induced by appropriately designed agonist ligands.

G protein-coupled receptors (GPCRs) represent a major class of membrane-bound proteins that mediate a wide variety of biological functions, including sensitivity to light and odors, endocrine and cardiovascular control, and neurotransmission. The ligand-induced intracellular signaling of GPCRs is tightly regulated by several mechanisms. For numerous GPCRs, desensitization involves translocation of arrestins from the cytosol to the cell membrane, their direct interaction with agonist-activated GPCRs, and consequent inhibition of G protein coupling (1, 2). Additionally, arrestin-mediated internalization of agonist-receptor complexes through clathrin-coated vesicles and subsequent receptor recycling to the cell membrane are responsible for the recovery of cellular responsiveness to agonists (resensitization). Receptor desensitization and resensitization accomplish the fundamental physiological role of modulating the cellular responses to both acute and chronic stimulation. An important implication is that not only signal transduction per se, but also the mechanisms regulating signal transduction have a profound influence in determining the pathophysiological processes mediated by GPCRs. Many important questions remain regarding the relationship between receptor occupancy, signaling, and desensitization. In particular, the structural features that differentiate the active and desensitized states and how extracellular interactions with the ligand translate into intracellular events (such as G protein activation and interaction with arrestins) have not been fully characterized.

Parathyroid hormone (PTH) is a major regulator of serum calcium homeostasis and bone metabolism (3). PTH-related protein (PTHrP), first described as the hormone responsible for hypercalcemia of malignancy, is now recognized as an autocrine/paracrine factor with various biological functions in many tissues (4). Both PTH and PTHrP bind to and activate the PTH type 1 receptor (PTH1Rc), a member of the class II subfamily of GPCRs coupled to both Gαq and Gαi proteins (5). As for many other GPCRs, interaction of activated PTH1Rc with β-arrestin-2 is a primary mechanism for rapid desensitization of cAMP signaling and further internalization of ligand-receptor complexes (6, 7). Several lines of evidence suggest that recruitment of β-arrestin-2 to the cell membrane and its association with activated PTH1Rc are largely (if not completely) independent of intracellular signaling and receptor phosphorylation (7–9).

PTH type 1 receptor; Bpa, p-benzoylphenylalanine; Bpa1-PTHrP-(1–36), [Bpa1,ile6,Arg11,13,Tyr36]PTHrP-(1–36)-NH2; Rho-Bpa1-PTHrP-(1–36), [Bpa1,ile6,Arg11,13,N′-5-carboxymethylrhodamine], Tyr36[Rho-PTHrP-(1–36)-NH2; Nle, norleucine; Nal, naphthylalanine; Rho-PTH-(1–34), [Nle1, lty6,N′-5-carboxymethylrhodamine], 1,2-Nal2, Arg23, Tyr34[PTH-(1–34)-NH2; HPLC, high pressure liquid chromatography; HEK, human embryonic kidney; GFP, green fluorescent protein; β-arrestin-2, β-arrestin-2; TM, transmembrane domain; IC3, third intracellular loop.

This paper is available on line at http://www.jbc.org
Therefore, the increased affinity of agonist-occupied PTH1Rc for β-arrestin-2 and the consequent reduction in coupling with G proteins may result from specific conformational states distinct from those of the active G protein-bound receptor. To characterize the specific interactions with agonists that stabilize these distinct functional states, we developed PTHrP (i.e., PTHrP-(1–36)) with selective pharmacological profiles. Analysis of the signaling properties of these analogs combined with fluorescence monitoring of the cellular distribution and extensive molecular modeling of the ligand-receptor complexes allowed us to identify specific interactions between ligand and receptor and the related conformational changes within PTH1Rc that are associated with β-arrestin-2-mediated desensitization.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Radioligand Preparation**—The synthesis, purification, and characterization of PTHrP-(1–34)-NH2 (PTHrP-(1–34)), PTHrP-(2–36)-NH2 (PTHrP-(2–36)), Bpa1-[Nle16,Arg17, Tyr26]PTHrP-(1–36)-NH2 (Bpa1-PTHrP-(1–36)), Bpa1-PTHrP-(1–36), Bpa1-[Nle16,Arg17, Tyr26, Lys19]PTHrP-(1–36)-NH2 (Bpa1-PTHrP-(1–36), bovine [Nle13,Tyr14]PTH-(33–34)-NH2 (PTH-(33–34), and bovine [Nle6,15,Lys9,N5-carboxymethylrhodamine]-1,2-Na21[^27], Arg26,Ty26[PH-(34–34)-NH2 (Rho-PTH-(1–34)) were carried out as previously described (6). The purity products were characterized by analytical HPLC, electron spray mass spectrometry, and amino acid analysis. Radiiodination and HPLC purification of PTH-(1–34), PTHrP-(2–36), and Bpa1-PTHrP-(1–36) were carried out as reported (6).

**Cell Culture and Transfection Method**—Human embryonic kidney cells (HEK-293) and HEK-293 cells stably expressing human PTH1Rc (clone C-21) were cultured in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum as described (10). Cells were plated at 1.5 × 10^5 cells/coverslip on 35-mm glass coverslips for fluorescence microscopy experiments and at 1.0 × 10^5 cells/well in 24-well plastic multiwell plates (Corning Inc., Corning, NY) for adenylyl cyclase, radioligand binding and internalization assays and transfected as previously described (7). All subsequent experiments were performed 24 h after transfection.

**Radioreceptor Binding and Internalization Assays**—Radioreceptor binding and internalization assays were carried out as reported (7) using HPLC-purified radiolabeled compounds. The binding affinities (Kd) for each analog were calculated from Scatchard analysis of radioreceptor binding assays (using each radiolabeled compound as tracer and competition with each unlabeled analog) as described previously (6, 7).

**Adenyl Cyclase, Intracellular Calcium, and Inositol Phosphate Assays**—cAMP accumulation was determined in subconfluent cell cultures of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (3-iM)-sensitive HEK-293 cells stably expressing Bpa1-PTH-(1–36), with antagonist/inverse agonist properties (16). In contrast, modification of residue 1 (Ala) in PTHrP-(1–36) by either deletion or substitution with Bpa (yielding PTHrP-(2–36) and Bpa1-PTHrP-(1–36), respectively) did not significantly alter the affinity for PTH1Rc or the efficacy in acutely stimulating the Gβγ/adenylyl cyclase signaling pathway in HEK-293 cells stably expressing human PTH1Rc (C-21 cells) (Table 1). However, although PTH-(1–34) and PTHrP-(1–36) stimulated rapid and robust transient increases in intracellular calcium in C-21 cells, both PTH-(2–36) and Bpa1-PTHrP-(1–36) lost their ability to stimulate Gβγ phospholipase C, as evidenced by both the absence of intracellular calcium transients upon stimulation of PTH1Rc in C-21 cells (Table 1) as well as the lack of stimulation of phosphatidylinositol hydrolysis (see Fig. 3C).

**Cellular Distribution of Ligands, PTH1Rc, and β-Arrestin-2**—Using β-2-0.02 (28), the translocation of β-arrestin-2 from the cytosol to the cell membrane and its colocalization with agonist-occupied receptors were monitored in C-21 cells. In contrast to native PTHrP-(1–36) and PTHrP-(1–34) (6, 7), neither Bpa1-PTHrP-(1–36) nor PTHrP-(2–36) induced detectable β-arrestin-2-GFP mobilization (Fig. 1). Furthermore, a rhodamine-
posed "pseudo-irreversible" binding of agonist to receptor-G
ing by PTH1Rc (7), we hypothesized that Bpa1-PTHrP-(1–36)
883 and /H11001 (50x30)
fully responsive to restimulation with these agonists after 2 h
over, cells pretreated with PTHrP-(1–36) and PTH-(1–34) were
36) (Fig. 3
53.8 ± 3.9
40.9 ± 3.9
43.5 ± 1.8
39.0 ± 3.6
46.0 ± 2.0
ND
ND

| Ligand | K<sub>D</sub><sup>a</sup> | EC<sub>50</sub><sup>b</sup> | Maximal acute cAMP (×fold increase over base line ± S.E.) | Residual cAMP (×fold increase over base line ± S.E.) | Re-challenged cAMP (×fold increase over base line ± S.E.) | Intracellular calcium transients<sup>c</sup> |
|--------|---|---|-----------------|-----------------|-----------------|------------------|
| PTH-(1–34) | 1.2 ± 0.2 | 2.5 ± 0.1 | 48.2 ± 3.9 | 5.3 ± 0.2 | 42.2 ± 2.3 | 150 nM |
| PTHrP-(2–36) | 1.0 ± 0.1 | 2.0 ± 0.1 | 75.0 ± 2.4 | 34.3 ± 2.5 | 150 nM |
| Bpa<sub>1</sub>-PTHrP-(1–36) | 14.3 ± 2.0 | 4.0 ± 0.2 | 40.9 ± 3.9 | 40.9 ± 3.9 | 40.9 ± 3.9 | ND |
| PTHrP-(2–36) | 2.0 ± 0.3 | 3.5 ± 0.2 | 43.5 ± 1.8 | 39.0 ± 3.6 | 46.0 ± 2.0 | ND |

<sup>a</sup>Binding affinity (K<sub>D</sub>) was calculated from Scatchard analysis of radioreceptor binding assays (using each radioiodinated compound as tracer and competition with each unlabeled agonist) performed in triplicate (6, 7).

<sup>b</sup>EC<sub>50</sub> was calculated from dose-response curves for cAMP accumulation. Cells were incubated with various concentrations of ligands (10<sup>-11</sup> to 10<sup>-6</sup>) for 15 min at 37 °C in the presence of 1 mM 3-isobutyl-1-methylxanthine. cAMP was measured as previously described (6, 7).

<sup>c</sup>To evaluate residual cAMP accumulation, cells were incubated for 30 min with 100 nM ligands, followed by three washes with phosphate-buffered saline and a 2-h incubation at 37 °C in cell culture medium containing [3H]adenine. At the end of this period, 1 mM 3-isobutyl-1-methylxanthine was added for 30 min, and cAMP was measured as previously described (6, 7). The basal cAMP level was 650 ± 37 cpm/well.

<sup>d</sup>To evaluate maximal cAMP accumulation after re-challenge with the agonist, cells previously incubated with the ligand, washed, and incubated for 2 h, as described above, were restimulated for 15 min with the agonist in the presence of 1 mM 3-isobutyl-1-methylxanthine. The basal cAMP level was 650 ± 37 cpm/well.

<sup>e</sup>Intracellular calcium transients were measured spectroscopically in Fura-2-loaded cells stimulated with 100 nM ligands as reported previously (10). ND, non-detectable generation of calcium transients.

line-labeled Bpa<sub>1</sub>-PTHrP-(1–36) analog that bound to PTH1Rc
on the cell membrane did not colocalize with β-arrestin-2-GFP on the cell surface or intracellularly (Fig. 2A). Rapid receptor internalization did not occur in response to the modified PTHrP analogs in cells expressing PTH1Rc-GFP (data not shown) (29), and Bpa<sub>1</sub>-PTHrP-(1–36)-receptor complexes were not detectable intracellularly (Fig. 2B). These results, distinct from those obtained with the full-length agonists PTH-(1–34) and PTHrP-(1–36) (Fig. 2, A and B) (6, 7), indicate that the G<sub>s</sub>-selective agonists PTH-(1–34) and Bpa<sub>1</sub>-PTHrP-(1–36) are unable to stimulate translocation of β-arrestin-2 to the cell membrane, β-arrestin-2 interaction with agonist-occupied PTH1Rc, and subsequent ligand-PTH1Rc complex endocytosis.

Radioligand Distribution—To quantify and confirm these observations, the distribution of radioiodinated PTH-(1–34), PTHrP-(2–36), and Bpa<sub>1</sub>-PTHrP-(1–36) was monitored (Fig. 3A); <sup>125</sup>I-PTH-(1–34) was rapidly (%:
10 min) and efficiently internalized in C-21 cells, whereas internalization of <sup>125</sup>I-Bpa<sub>1</sub>-PTHrP-(1–36) and <sup>125</sup>I-PTHrP-(2–36) was slower (%: 30 min) and only partial. In addition, the dissociation of receptor-bound <sup>125</sup>I-Bpa<sub>1</sub>-PTHrP-(1–36) and <sup>125</sup>I-PTHrP-(2–36) (14 ± 1 and 16 ± 2% at 60 min, respectively) was lower than that of <sup>125</sup>I-PTH-(1–34) (20 ± 6% at 60 min), in agreement with the proposed “pseudo-irreversible” binding of agonist to receptor-G protein complexes (30). As a consequence, 1 h after extensive washout, both Bpa<sub>1</sub>-PTHrP-(1–36) and PTHrP-(2–36) remained mostly associated with PTH1Rc on the cell membrane (%: 47 ± 5 and 61 ± 3% of total bound ligand, respectively), whereas only 10 ± 1% of PTH-(1–34) was still associated with membrane receptors.

PTH1Rc Desensitization and Resensitization of cAMP Signaling—Because receptor interaction with β-arrestin-2 is an essential early step in rapid desensitization of G<sub>s</sub>-cAMP signaling by PTH1Rc (7), we hypothesized that Bpa<sub>1</sub>-PTHrP-(1–36) and PTHrP-(2–36) would induce sustained activation of adenyl cyclase. Indeed, exposure of HEK cells expressing PTH1Rc either stably (C-21) or transiently to Bpa<sub>1</sub>-PTHrP-(1–36) or PTHrP-(2–36) for 30 min, followed by a 2-h washout, resulted in a 5–7-fold increase in residual levels of cAMP accumulation at the end of this period compared with cells similarly exposed to PTHrP-(1–36) or PTH-(1–34) (Fig. 3B and Table I). Moreover, cells pretreated with PTHrP-(1–36) and PTH-(1–34) were fully responsive to re-stimulation with these agonists after 2 h (+883 and +796% above residual levels, respectively), indicat-

FIG. 1. Fluorescence microscopy monitoring of β-arrestin-2 mobilization in response to PTHrP analogs. HEK-293/C-21 cells stably expressing human PTH1Rc were transiently transfected with β-arrestin-2-GFP (0.3 μg of DNA35-mm glass coverslip) as previously described (6, 7). In the absence of stimulation, β-arrestin-2-GFP was distributed throughout the cytoplasm (panel a). Stimulation for up to 40 min at 37 °C of the cells shown in panel a with 100 nM PTHrP-(2–36) did not mobilize β-arrestin-2-GFP (panel b). A similar lack of β-arrestin-2-GFP mobilization was observed with 100 nM Bpa<sub>1</sub>-PTHrP-(1–36) (panel c). In contrast, 100 nM PTHrP-(1–36) recruited β-arrestin-2-GFP to the cell membrane within 5 min (panel d). Each panel is representative of eight independent experiments yielding similar results.

Rescue of Signaling Regulation and Endocytosis by the PTH1Rc(H223R) Mutant—To elucidate whether such critical
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Fig. 2. Cellular colocalization of ligand, PTH1Rc, and arrestin-2 in response to PTHrP analogs. A, HEK-293 cells transiently coexpressing human PTH1Rc and β-arr2-GFP were incubated with 50 nM Rho-Bpa1-PTHrP-(1–36) (panels a–c) or Rho-PTH-(1–34) (panels d–f). After 15 min at 37 °C, Rho-Bpa1-PTHrP-(1–36) appeared bound on the cell surface without evidence of internalization (panel a); β-arr2-GFP was not recruited to the cell surface (panel b); and therefore, the two fluorescences did not colocalize on the overlay image (panel c). In contrast, after stimulation with Rho-PTH-(1–34), both ligand (panel d) and β-arr2-GFP (panel e) colocalized intracellularly (panel f). B, HEK-293 cells transiently expressing human PTH1Rc-GFP were incubated with 50 nM Rho-Bpa1-PTHrP-(1–36) (panels a–c) or Rho-PTH-(1–34) (panels d–f). After 15 min at 37 °C, Rho-Bpa1-PTHrP-(1–36) was linearly distributed on the cell surface (panel a); PTH1Rc-GFP was mostly localized on the cell surface (panel b); and therefore, both ligand and receptor colocalized (in orange-yellow in the overlay image) (panel c). In contrast, in response to Rho-PTH-(1–34), both ligand (panel d) and receptor (panel e) were internalized and colocalized in the cytoplasm (panel e). Each panel is representative of three to seven independent experiments yielding similar results.

differences between PTHrP and its analogs modified at position 1 were dependent on differences in ligand-induced receptor conformation or Gq-mediated signaling, we investigated whether regulation of cAMP signaling and cellular distribution of Bpa1-PTHrP-(1–36) with β-arr2-GFP could be rescued by the PTH1Rc(H223R) mutant. Indeed, this natural mutant is Gq signaling-deficient both in COS-7 cells (31) and in HEK-293 cells, where no measurable generation of inositol phosphates in response to PTHrP-(1–36), Bpa1-PTHrP-(1–36), or PTHrP-(2–36) was detected (Fig. 3C). Furthermore, in HEK-293 cells, PTH1Rc(H223R) is constitutively associated with β-arrestin-2 on the cell membrane and internalizes normally in response to PTH-(1–34) and PTHrP-(1–34) (7). In this system, brief exposure to Bpa1-PTHrP-(1–36) and PTHrP-(2–36), followed by a 2-h washout, did not result in cAMP levels higher than the basal activity, which is reflective of the constitutive activity of the receptor mutant (Fig. 3B). Moreover, 2 h after the first agonist stimulation, PTH1Rc(H223R)-expressing cells were fully responsive to a re-challenge with agonist (Fig. 3B), indicating that the elevated constitutive activity in these cells is not a limiting factor for the ligand-induced generation of cAMP. This was accompanied by internalization of 125I-Bpa1-PTHrP-(1–36) and 125I-PTHrP-(2–36) bound to PTH1Rc(H223R) (68 ± 3 and 54 ± 3%, respectively), which was similar to 78 ± 2% for 125I-PTH-(1–34). Additionally, rhodamine-labeled Bpa1-PTHrP-(1–36) colocalized with PTH1Rc(H223R)-associated β-arr2-GFP on the cell membrane and then intracellularly (Fig. 3D), as was previously seen with rhodamine-labeled PTH-(1–34) (7). These experiments show that the absence of activation of the Gq/phospholipase C signaling pathway is not responsible for lack of rapid cAMP desensitization of wild-type PTH1Rc in response to PTHrP analogs. Rather, these data indicate that interaction of the receptor with β-arrestin-2 is sufficient for the regulation of Gq/cAMP signaling.

Molecular Modeling of Ligand-PTH1Rc Complexes—To further explore the structural details underlying these effects, we examined the conformational changes induced by binding of native PTHrP-(1–36) and its position 1-modified analogs to human PTH1Rc using molecular dynamics simulations. The starting ligand-receptor structure, based on extensive NMR characterization of the ligands and extra- and intracellular portions of PTH1Rc, also includes ligand-receptor contact points identified by photoaffinity labeling experiments (16–23). Among these, of particular significance is the interaction of position 1 of Bpa1-PTHrP-(1–36) with Met420 at the extracellular end of TM6 of PTH1Rc (16).

Analysis of simulations with PTHrP-(2–36) and Bpa1-PTHrP-(1–36) indicated significant differences from simulations with the native hormone, PTHrP-(1–36) (Fig. 4), in particular with regard to the orientation of TM5 and TM6. The root-mean-square deviation for TM5–TM6 was 3.5 Å between the wild-type ligand and Bpa1-PTHrP-(1–36). A much smaller value of 1.5 Å was obtained for the remaining helices (TM1–TM4 and TM7). This resulted in major effects on the structure of the third intracellular loop (IC3), particularly the N-terminal helix (Thr387–Arg396) of the intracellular loop. With PTHrP-(1–36), this helix was found to be parallel to the membrane surface, leading to the exposure of the charged residues, Lys408, Arg409, and Glu410. In contrast, with the Gq-selective modified ligands, the helix-turn-helix structure of the third loop, as observed in our NMR studies (23, 32), was maintained. In all of the simulations, smaller effects on the orientation of the C-terminal portion of IC3 were observed. These changes, mostly evident with Lys408, were identical to those observed for PTH1Rc mutants that constitutively activate adenylyl cyclase (14). The results for PTHrP-(2–36) were very similar to those for Bpa1-PTHrP-(1–36), suggesting that the first residue of PTHrP is actively involved in the induction of the receptor conformation recognized by β-arrestin-2.

DISCUSSION

The objective of this study was to characterize the structural and functional consequences of modifications affecting the first amino acid residue in PTHrP and thereby to provide new insights into the PTH1R conformational states associated with coupling to G proteins and desensitization of intracellular signaling. Our data clearly show that modifications of residue 1 in the biologically active N-terminal fragment of PTHrP result in ligands (i.e. PTHrP-(2–36) and Bpa1-PTHrP-(1–36)) that, although maintaining high affinity and efficacy in stimulating cAMP production, are unable to stimulate the Gq/phospholipase C signaling pathway. Additionally, in contrast to native PTHrP and PTH (6–9), the modified PTHrP analogs do not stimulate β-arrestin-2 translocation to the cell membrane and
FIG. 3. A, internalization of radiolabeled PTH-(1–34), Bpa1-PTHrP-(1–36), and PTHrP-(2–36) in HEK-293/C-21 cells. HEK-293/C-21 cells stably expressing PTH1Rc were incubated for 2 h at 4 °C with the radiolabeled agonist 125I-PTH-(1–34) (●), 125I-Bpa1-PTHrP-(1–36) (▲), or 125I-PTHrP-(2–36) (●); washed; and incubated in fetal bovine serum-supplemented Dulbecco's minimal essential medium for the indicated times. The percentage of specific radioligand internalization was evaluated as reported (6, 7). Each point represents the mean percentage ± S.E. of total cell-associated ligand from triplicate determinations. Similar results were obtained in three additional experiments. Average values of all experiments for internalization at 60 min were as follows: PTH-(1–34), 71 ± 9% of the total cell-associated radioligand; Bpa1-PTHrP-(1–36), 37 ± 6% (p < 0.001 compared with PTH-(1–34)); and PTHrP-(2–36), 25 ± 7% (p < 0.001 compared with PTH-(1–34)).

B, sustained adenylyl cyclase activity by position 1-modified PTHrP analogs is inhibited by the PTH1Rc(H223R) mutant. HEK-293 cells transiently expressing either wild-type PTH1Rc (white and thinly hatched bars) or the constitutively active PTH1Rc(H223R) mutant (black and thickly hatched bars) (0.4 µg of DNA/well in 24-well plates) were treated with the indicated ligands (100 nM) for 30 min, followed by a 2-h washout at 37 °C. Residual and re-challenged cAMP accumulation was then measured for 30 min in the presence of 3-isobutyl-1-methylxanthine (1 mM) without (white and black bars) and with re-exposure (hatched bars) to PTHrP or its modified analogs, respectively. In cells expressing wild-type PTH1Rc, residual cAMP accumulation after preincubation with Bpa1-PTHrP-(1–36) and PTHrP-(2–36) was significantly higher than after preincubation with PTHrP-(1–34). Residual cAMP accumulation after preincubation with Bpa1-PTHrP-(1–36) and PTHrP-(2–36) was lower (p < 0.001) in cells expressing PTH1Rc(H223R) than in cells expressing wild-type PTH1Rc. Also, in cells expressing PTH1Rc(H223R), the response to a re-challenge with the agonist (p < 0.01 compared with residual cAMP levels) was similar to that in cells not previously treated with the ligand. The maximal cAMP accumulation in these cells, evaluated by stimulation of adenylyl cyclase with forskolin (10 µM), was 24,027 ± 947 cpm/well. Results are presented as means ± S.E. from three independent experiments performed in triplicate.

C, inositol phosphate (IP) generation by PTHrP-(1–34), Bpa1-PTHrP-(1–36), and PTHrP-(2–36) in HEK-293 cells expressing wild-type PTH1Rc or PTH1Rc(H223R). HEK-293 cells transiently expressing either wild-type PTH1Rc (white bars) or the PTH1Rc(H223R) mutant (gray bars) (0.4 µg of DNA/well in 24-well plates) were loaded with myo-[3H]inositol and treated with the indicated ligands (1 µM) for 60 min at 37 °C in the presence of 20 mM LiCl. In cells expressing wild-type PTH1Rc, only PTHrP-(1–34) induced generation of inositol phosphates (p < 0.001 versus basal activity). Cells expressing the PTH1Rc(H223R) mutant were unresponsive to all ligands. Results are expressed as means ± S.E. from two independent experiments performed in triplicate. Basal values were subtracted from the values obtained in the presence of the hormone. D, rescue of Bpa1-PTHrP-(1–36) internalization by PTH1Rc(H223R). HEK-293 cells cotransfected with the PTH1Rc(H223R) mutant and β-arrestin-2-GFP were incubated for 10 min at room temperature with Rho-Bpa1-PTHrP-(1–36) (50 nM). In these cells, β-arrestin-2-GFP was constitutively recruited to the cell membrane (panel a). The ligand at first appeared to be linearly bound on the cell surface (panel b) and colocalized with β-arrestin-2-GFP (in yellow in the overlay image) (panel c). After 15 min at 37 °C, both β-arrestin-2-GFP (panel d) and the fluorescent ligand (panel e) were internalized and colocalized in the cytoplasm (panel f).
its association with agonist-activated PTH1Rc, functionally resulting in impaired desensitization of cAMP signaling. The observation that regulation of signaling by PTHrP-(2–36) and Bpa1-PTHrP-(1–36) and ligand-receptor endocytosis were rescued by the mutant receptor PTH1Rc(H223R), which is defective in Gq-mediated signaling (31) but is constitutively associated with β-arrestin-2 (7), clearly indicates that the signaling selectivity of these analogs per se is not the cause of the impaired desensitization. Rather, when combined with the findings that receptor phosphorylation is not necessary for β-arrestin-2 translocation (7, 8) and with the previous characterization of the molecular motions of the PTH1Rc(H223R) mutant showing that the conformation of the third intracellular loop of the unbound receptor mutant is very similar to the one of cognate agonist-bound PTH1Rc (14), these experiments suggest that the known interaction between residue 1 of PTHrP and the extracellular end of transmembrane helix VI of PTH1Rc (16) mediates the conformational changes leading to the high affinity state for β-arrestin-2. In structural terms, interaction of Bpa1-PTHrP-(1–36) and PTHrP-(2–36) with PTH1Rc results in stabilization of a non-desensitized G protein-coupled conformation. In contrast, complexes with native PTHrP-(1–36) allow for a distinct confor-
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J. Biol. Chem. 2002, 277:38524-38530.
doi: 10.1074/jbc.M202544200 originally published online July 9, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M202544200

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