Identification of Determinants of Inverse Agonism in a Constitutively Active Parathyroid Hormone/Parathyroid Hormone-related Peptide Receptor by Photoaffinity Cross-linking and Mutational Analysis

We have investigated receptor structural components responsible for ligand-dependent inverse agonism in a constitutively active mutant of the human parathyroid hormone (PTH)/parathyroid hormone-related peptide (PTHrP) receptor type 1 (hP1R). This mutant receptor, hP1R-H223R (hP1R CAM-HR), was originally identified in Jansen’s chondrodysplasia and is altered in transmembrane domain (TM) 2. We utilized the PTHrP analog, [Bpa2,Leu3,Trp23,Tyr36]PTHrP-(1–36)-amide (Bpa2-PTHrP-(1–36)), which has valine 2 replaced by p-benzoyl-L-phenylalanine (Bpa); this substitution renders the peptide a photoreactive inverse agonist at hP1R CAM-HR. This analog cross-linked to hP1R CAM-HR at two contiguous receptor regions as follows: the principal cross-link site (site A) was between receptor residues Pro415–Met441, spanning the TM6/extracellular loop three boundary; the second cross-link site (site B) was within the TM4/TM5 region. Within the site A interval, substitution of Met425 to Leu converted Bpa2-PTHrP-(1–36) from an inverse agonist to a weak partial agonist; this conversion was accompanied by a relative shift of cross-linking from site A to site B. The functional effect of the M425L mutation was specific for Bpa2-containing analogs, as inverse agonism of Bpa2-PTH-(1–34) was similarly eliminated, whereas inverse agonism of [Leu1,d-Trp23]PTHrP-(5–36) was not affected. Overall, our data indicate that interactions between residue 2 of the ligand and the extracellular end of TM6 of the hP1R play an important role in modulating the conversion between active and inactive receptor states.

The biological effects of the calcium regulatory hormone PTH and the paracrine factor PTHrP are mediated through the PTH/PTHrP receptor (P1R), a family B G-protein-coupled receptor. The amino-terminal fragments PTH-(1–34) and PTHrP-(1–36) bind to the P1R with high affinity (Kd = 3 nM) and activate this receptor with full efficacy (1, 2). Despite having only limited amino acid sequence homology that is restricted to the first 13 residues, both ligands have similar functional domains; the amino-terminal portion (residues 1–14) is important for receptor activation, whereas the carboxy-terminal portion (residues 15–34) is important for high affinity receptor binding (3). Cross-linking and mutagenesis studies suggest that the activation domain of the ligand interacts with the juxtamembrane portion of the receptor composed of the extracellular loops and the extracellular ends of the TM helices, and that the binding domain of the ligand interacts with the amino-terminal extracellular domain of the receptor (4–7). Consistent with this model, certain amino-terminal modifications or deletions in either PTH or PTHrP result in analogs that act as P1R antagonists (8–10).

Inverse agonists are ligands that reduce receptor signaling activity to below the basal signaling level seen with the unoccupied receptor (11, 12). These compounds are thus functionally different from agonists, which activate the receptor, and neutral antagonists, which have no efficacy of their own but can prevent the actions of both agonists and inverse agonists by a simple competitive mechanism (13). At least some constitutive activity of a receptor is required in order for inverse agonist activity to be detected; in general, such constitutive activity may be induced by certain receptor mutations or by overexpressing the wild-type form of a receptor (14–16). By using either method to increase basal receptor signaling, many ligands that were previously classified as neutral antagonists were subsequently shown to be inverse agonists (14–16). Some of these ligands have gained clinical significance, for example as histamine-1 blockers (17), histamine-2 blockers (18), beta-blockers (19), or antidepressants (16, 20). How the inverse agonist activities of these compounds relate to their clinical effectiveness has not yet been determined. Furthermore, the molecular mechanisms by which inverse agonists reduce receptor signaling activity remain largely unknown, although the pharmacological behavior of these ligands has been discussed in theoretical terms (12, 13, 21). One crucial question is whether or not the receptor contact points that mediate inverse agonism are distinct from those that induce receptor activation.

Jansen’s chondrodysplasia is a rare human disease caused by mutations in the P1R that result in constitutive activity (22). Patients with this disorder have skeletal abnormalities, hypercalcemia, and low serum PTH and PTHrP levels; clinical manifestations that are consistent with the important role that the P1R plays in skeletal development and calcium homeostasis (23). Three different P1R mutations have been identified in these patients: His223 → Arg (hP1R CAM-HR), Thr413 → Pro

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The abbreviations used are: PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; Bpa, p-benzoyl-L-phenylalanine; TM, transmembrane domain; P1R, PTH/PTHrP receptor; PAGE, polyacrylamide gel electrophoresis; Tricine, N-2-hydroxy-1,1-bis(hydroxymethyl) ethyglycine.

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agonist at hP1RCAM-HR, as it is at the wild-type hP1R. The structur-
al23,Arg 13,26,27, Tyr 34] bovine PTH-(1
contains a photolabile amino acid derivative (Bpa2-PTHrP-(1
36)-amide, cross-links at or near Met425, in the extracellular
site for Bpa2-PTHrP-(1
36)-amide (Bpa2-PTH-(1
36)-amide and [n-PTH-(7-34)] hPTH(7-34-amide) (27). Recently, we
reported (28) the characterization of several new PTH and PTHrP
antagonists that act as inverse agonists at each of the
constitutively active P1R mutants. One of these analogs,
[Bpa2,Ille,Trp23, Tyr36]PTHrP-(1-36)-amide (Bpa2-PTHrP-(1-36)),
contains a photolabile amino acid derivative (p-benzoyl-l-phenyl-
alanine) at position 2 and functions as an inverse agonist at
hP1RCAM-HR; however, it is not an inverse agonist at hP1RCAM-TP
or hP1RCAM-IR, and it is a weak partial agonist at the wild-type
receptor (28). Importantly, the photolabile position 2 amino acid
derivative itself is responsible for the inverse agonist activity of this
analog at hP1RCAM-HR, as the otherwise isosteric parent peptide
[Ile5,Trp23, Tyr36] PTHrP-(1-36)-amide (valine at position 2) is an
agonist at hP1RCAM-HR as it is at the wild-type hP1R. The structural
requirements for inverse agonist activity at position 2 in
PTHrP-(1-36) are highly specific, because none of several other
position 2 modifications that are structurally similar to Bpa,
including tryptophan and the ε-Bpa stereoisomer, confer inverse
agonism to the peptide ligand (28).

Recent photoaffinity mapping studies performed with the
wild-type hP1R have indicated that the benzophenone group of
a similar antagonist analog, [Bpa2,Ille, Arg1,13, Tyr39]PTH-(1
36)-amide, cross-links at or near Met425, in the extracellular
term of TM6 (6). We hypothesized that mapping the cross-linking
site for Bpa2-PTHrP-(1-36) in hP1RCAM-HR would help identify
receptor residues that play a role in mediating the inverse
agonist activity of this analog, as well as residues that play a
role in converting the receptor between active and inactive
conformations. Thus, we physically mapped the cross-linking
site of Bpa2-PTHrP-(1-36) in hP1RCAM-HR. We also showed by
mutational methods that a residue within the mapped receptor
interval is involved in mediating the inverse agonist effect of
Bpa2-PTHrP-(1-36).

**EXPERIMENTAL PROCEDURES**

**Materials**—[Ile8,Trp23, Tyr36] PTHrP-(1-36)-amide (PTHrP-(1
36)), [Bpa2,Ille,Trp23, Tyr36] PTHrP-(1-36)-amide (Bpa2-PTHrP-(1-36)),
[Nle11, Tyr1] rPTH-(3-34)-amide (rPTH-(3-34)), [Bpa2,Nle18, Na-
l23,Arg13,26,27, Tyr34] bovine PTH(1-34)-amide (Bpa2-PTH-(1-34)),
[Nle11, Tyr1] bPTH-(3-34)-amide (bPTH-(3-34)), [Ile15,Ille,Trp23,
Trp39] PTHrP-(5-36)-amide ([Leu11, i-TPr12, Trp23, Tyr39] PTHrP-(5-36))
were synthesized by the Protein and Peptide Core Facility at Massa-
chusetts General Hospital (Boston) by the solid-phase method on
PerkinElmer Life Sciences models 430A and 431A synthesizers. Pept-
idides were purified by reverse-phase high pressure liquid chromato-
graphy, and their compositions were confirmed by amino acid analysis and
mass spectroscopy.

**Cell Culture/DNA Transfection**—COS-7 cells were cultured in Dul-
beco’s modified Eagle’s medium supplemented with 10% heat-inacti-
ved fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin in a humidified atmosphere containing 95% air and 5%
 carbon dioxide. Cells were seeded in 24-well plates for radioreceptor
cross-linking experiments and 6-well plates for cell-based experiments. Once
the monolayer of COS-7 cells reached ~90% confluency, cells were transfected by the DEAE-dextran method as described (30) using 200
ng plasmid/well in 24-well plates or 800 ng of plasmid/well in 6-well
plates. After 4 days, cells were used for experiments.

**Partial Labeling of Peptides**—Radiolabeled peptides were prepared by
chemical-T amination, followed by high pressure liquid chromatog-
raphy purification using a 30–50% acetonitrile gradient in 0.1% triflu-
oracetic acid over 30 min.

**Radioligand-Receptor Binding and cAMP Accumulation Assays**—
Binding assays were performed as described previously (31). In brief, the
[Ile8]-labeled radioligand bPTH-(3-34) was incubated with cells ex-
pressing wild-type or mutant P1R in the presence of varying concen-
trations (0–10–4 M) of unlabeled peptide. After a 4-h incubation at
15°C, the binding mixture was removed, and the cells were lysed, and
the entire lysate was counted for γ-irradiation. Intracellular cAMP
accumulation after 30-min treatments at room temperature with or
without ligand was measured by radioimmunoassay as described previ-
ously (9).

**Photoaffinity Labeling of the P1R**—Cells transiently expressing wild-
type or mutant P1R were incubated with [125I]-labeled Bpa2-PTHrP-(1
36) (3 million cpm/well in 6-well plates) for 6 h at 4°C. Cells were rinsed
twice and covered with cold buffer. The plates were placed on an ice tray
under a UV light source (Blak Ray long-wave lamp, 366 nm, 7 milli-
 Watts/cm2; UV Products, San Gabriel, CA) at a distance of ~5 cm for 15
min. Cells were lysed using 1% Triton buffer and centrifuged at 1500 g
for 10 min. The supernatant was then mixed 1:1 with 2× SDS-PAGE
sample buffer to give final concentrations of 4% SDS, 80 mM Tris-HCl
(pH 6.8), 20% glycerol, 0.2% bromphenol blue, and 100 mM dithiothreitol.

**SDS-PAGE Analysis/Purification**—The samples in SDS-PAGE
sample buffer were incubated at room temperature for 2 h and then
subjected to SDS-PAGE analysis (10% acrylamide) performed according
to the method of Laemmli (32). For visualization of the intact cross-linking
complexes, wet gels were cut into strips and counted for γ-irradiation,
and the gel strips with peak counts were subjected to electroelution in
a dialysis bag (molecular mass cut-off = 12,000 Da) at 100 V for 2 h.
Eluted samples were concentrated using Centriplus-10 tubes (Millipore
corp., Bedford, MA).

**Chemical Cleavage and Size Analysis**—For cleavage of the receptor
at methionine residues (Fig. 1), the gel-purified radiolabeled ligand-
receptor complexes were incubated with CNBr (100 μg) in 70% formic
acid at 20°C for 1 h, followed by digestion with 200 units of trypsin
for 1 h at room temperature with or without ligand. The digestion
mixtures were dried in vacuo, redissolved in Laemmli sample buffer,
and subjected to autoradiography at ~80°C. For purification of cross-linked ligand-receptor
complexes, wet gels were cut into strips and counted for γ-irradiation,
and the gel strips with peak counts were subjected to electrophoresis in
a dialysis bag (molecular mass cut-off = 12,000 Da) at 100 V for 2 h.
Eluted samples were concentrated using Centriplus-10 tubes (Millipore
corp., Bedford, MA).

**Data Calculation**—Calculations were performed using Microsoft
Excel. Nonlinear regression analyses of binding and cAMP dose-response
dcurves were performed using the four-parameter equation: $y = \frac{Min + (Max - Min) \times 10^{\frac{x}{c}}}{1 + 10^{\frac{x}{c}}}$, where $Min = (Max - Min)/(1 + (IC_{50}/c)^{\beta})$. The Excel Solver function was utilized
for parameter optimization, as described previously (9, 34). Surface
receptor density ($B_{max}$) was calculated by the method of Scatchard
assuming a single class of binding site and equivalent binding of radi-
olabeled and unlabeled peptides. The statistical significance between
two data sets was determined using a two-tailed Student's t test, as-
suming unequal variances for the two sets.
RESULTS

Mapping of the Bpa²-PTHrP-(1–36) Cross-link Site—For both hP1R_CAM_HH and wild-type hP1R, cross-linking of Bpa²-PTHrP-(1–36) followed by SDS-PAGE autoradiography of the resulting COS-7 cell lysates yielded a single large (~79 kDa) protein band that was consistent with specific cross-linking to intact glycosylated PTH-1 receptors (Fig. 2A). No cross-linking was observed in COS-7 cells transfected with vector alone (Fig. 2A, lane 3). To map the cross-linked sites in the two receptors, we gel-purified the ligand-receptor complexes, treated the resulting complexes with cyanogen bromide (CNBr) in formic acid to cleave at the carboxyl-end of methionine residues (Fig. 1), size-separated the digestion products on tricine gels, and visualized the results by autoradiography.

In hP1R_CAM_HH, but not in hP1R, a third band of ~17 kDa was observed (Fig. 2B, lane 2).

The origin of the ~3.5-kDa band was assessed but proved to be refractory to identification. Treatment of the purified complexes with formic acid alone confirmed that the bulk of the radioactivity in this band arose from CNBr-dependent mechanisms (Fig. 2B, lanes 1 and 3). The mobility of the ~3.5-kDa band was indistinguishable from that of free radioligand (see Fig. 4). The release of free ligand by a CNBr-dependent cleavage process is known to occur if the ligand has cross-linked to the side chain methyl group of a methionine residue (35, 36). In fact, such CNBr-dependent release of free ligand was observed previously by others (6) for a related Bpa²-containing PTHR analog ([Bpa²,Ile⁶,Arg¹¹,¹³,Tyr³⁶]PTHrP-(1–36)-amide) cross-linked to the wild-type hP1R, and it was concluded in this study that cross-linking occurred to Met¹²⁵ because mutation of this residue to leucine markedly reduced cross-linking efficiency. In our current study, introduction of the Met¹²⁵ → Leu mutation in hP1R resulted in a moderate reduction in the yield of cross-linking product (Fig. 3A, lanes 3 and 4); however, when introduced into hP1R_CAM_HH, this same mutation resulted in a comparable if not greater yield of cross-linking product, relative to that obtained with the control hP1R_CAM_HH (Fig. 3A, lanes 1 and 2). Upon CNBr digestion, both hP1R-M425L and hP1R_CAM_HH-M425L complexes yielded a prominent ~3.5-kDa band (Fig. 3B). These results imply that within both hP1R and hP1R_CAM_HH at least some cross-linking occurred to a residue other than Met⁴²⁵ that can also yield free ligand upon CNBr digestion. We examined the possibility that Bpa²-PTHrP-(1–36) cross-linked to one of the other methionine residues in the juxtamembrane region (e.g. at positions 224, 231, 414, 441, and 445; cf. Fig. 1) by constructing hP1R mutants with these residues mutated individually or in combination. Each of the resulting mutant receptor complexes continued to show a prominent ~3.5-kDa band after CNBr digestion (Fig. 4); thus, this band could not be attributed to cross-linking of the ligand to the methyl group of any of the methionine residues tested. Whereas the origins of the ~3.5-kDa band remain uncertain, one possibility is that Bpa²-PTHrP-(1–36) cross-links to another oxidation-sensitive amino acid (e.g. Trp, Asn, Gln, and Tyr) that could be cleaved by CNBr, although such a mechanism has not previously been appreciated.

We next investigated the ~5.0-kDa band, which was observed prominently in the CNBr digestions of both the hP1R-Bpa²-PTHrP-(1–36) and hP1R_CAM_HH-Bpa²-PTHrP-(1–36) complexes. The size of this band was consistent with cross-linking of Bpa²-PTHrP-(1–36) to either of two predicted CNBr-generated fragments that are contiguous and within the juxtamembrane region of the receptor, Pro⁴¹⁵-Met⁴²⁵ and Ala⁴²⁶-Met⁴⁴¹. Mutation of Met⁴²⁵ → Leu in either hP1R_CAM_HH or hP1R, however, did not alter the mobility of the ~5.0-kDa band (Fig. 3B). A possible explanation for this observation was that CNBr did not cleave at Met⁴²⁵, potentially because of steric interference from the cross-linked Bpa²-PTHrP-(1–36). In this case, the cross-linked receptor fragment would consist of Pro⁴¹⁵→Met⁴⁴¹. The ~5.0-kDa band was no longer observed after CNBr digest of the complex formed with hP1R-M414V (Fig. 4B, lane 1), suggesting that M414V is indeed the amino-terminus of the cross-linked interval.

The CNBr digestion of the hP1R-M441L mutant receptor did not yield a detectable shift in the ~5.0-kDa band (Fig. 4A, lane 4), but this result could be explained by the proximity of the next methionine residue at position 445. Consistent with this explanation, the ~5.0-kDa band was not detected in the digest of the complex formed with a triple mutant receptor altered at Met⁴²⁵, Met⁴⁴¹, and Met⁴⁴⁵ (Fig. 4A, lane 2). Furthermore, in the CNBr digestions of the complexes formed with single mutants hP1R-M441L and hP1R-M445L (Fig. 4A, lanes 4 and 5) (as well as the triple mutant; Fig. 4A, lane 2), a faint ~15-kDa band could be detected; the size of this band correlated with the
ligand cross-linked to the Pro$^{415}$–Met$^{499}$ fragment (Fig. 1) and could be potentially attributed to failed cleavage at positions 441 and 445 in a minor fraction of these single mutant receptors. To test this possibility further, we introduced the double mutation of Met$^{441}$→Leu/Met$^{445}$→Ile into hP1R. After CNBr digestion of the complex formed with this mutant receptor, the −5.0-kDa band was no longer observed, and a new larger band of −15 kDa was detected. This new band correlated with the size predicted for Bpa$^{2}$-PTHrP-(1–36) cross-linked to the Pro$^{415}$–Met$^{499}$ fragment (Fig. 5A, lanes 1 and 2). These results thus established the identity of the −5.0-kDa band obtained with the wild-type hP1R as Bpa$^{2}$-PTHrP-(1–36) cross-linked to the receptor fragment Pro$^{415}$–Met$^{499}$

To determine the origin of the −5.0-kDa band in hP1R$_{CAM-HR}$, we examined the effect of the M414V single mutation as well as that of the double mutation M441L/M445I in this receptor on the CNBr digestion pattern. As shown in Fig. 5B (lane 2), the −5.0-kDa band was not observed in the CNBr digestion of the complex formed between Bpa$^{2}$-PTHrP-(1–36) and hP1R$_{CAM-HR}$-M414V, and the expected new band of −19 kDa was detected just above the −17-kDa band (as discussed below, in the digest of this mutant receptor, the −17-kDa band also showed a slight shift to −18 kDa). The new −19-kDa band correlate with the size predicted for Bpa$^{2}$-PTHrP-(1–36) cross-linked to the receptor fragment Ala$^{313}$–Met$^{441}$ (Fig. 1). Consistent with this interpretation, the −5.0-kDa band was no longer observed in the CNBr digestion of the complex formed with hP1R$_{CAM-HR}$-M441L/M445I (Fig. 5C, lanes 1 and 2). In these digests, the expected new larger band of −15 kDa was not detected; nevertheless, the combined results confirmed that, as with hP1R, the −5-kDa band obtained with hP1R$_{CAM-HR}$ corresponds to Bpa$^{2}$-PTHrP-(1–36) cross-linked to the receptor fragment Pro$^{415}$–Met$^{499}$

The size of the −17-kDa band observed in the CNBr digests of hP1R$_{CAM-HR}$ was most consistent with Bpa$^{2}$-PTHrP-(1–36) cross-linked to the receptor segment Ala$^{313}$–Met$^{441}$ (Fig. 1). The apparent shift of this −17 to −18 kDa seen in the digest of the hP1R$_{CAM-HR}$-M414V mutant receptor described above (Fig. 5B, lanes 1 and 2) verified this assignment, as it confirmed that Met$^{414}$ was a boundary residue for the cross-linked receptor interval. The overall mapping results obtained with hP1R$_{CAM-HR}$ therefore suggest that Bpa$^{2}$-PTHrP-(1–36) can cross-link to either of two sites in two contiguous intervals of the juxtamembrane region of the receptor; one site (site A) is delimited by residues Pro$^{415}$–Met$^{441}$ and gives rise to the −5.0-kDa band, and the other (site B) is delimited by residues...
Ala^{313}–Met^{414} and gives rise to the −17-kDa band. In addition, the observation that the M414V mutation in hP1R CAM-HR did not result in a single merged receptor fragment band of −19 kDa (Ala^{313}–Met^{441}) but instead produced this band together with an −18-kDa band (Ala^{313}–Met^{425}; Fig. 5B) indicated that CNBr could cleave at Met^{425} in those receptors in which cross-linking occurred to site B, but not in those in which cross-linking occurred to site A.

Methionine 425 Is a Receptor Determinant of Bpa²-PTHrP-(1–36) Inverse Agonist Activity—The failed CNBr cleavage at Met^{425} that we observed when Bpa²-PTHrP-(1–36) was cross-linked to Met^{425} in those receptors in which cross-linking occurred to site A (e.g. within the Pro^{415}–Met^{441} segment) suggested that cross-linking occurred at or near this residue. Moreover, mutation of Met^{425} to Leu in hP1RCAM-HR resulted in a reduction in the relative amount of cross-linking to site A and an increase in the relative amount of cross-linking to site B (Fig. 3B, lanes 1–2), a result which indicates that the residue at

FIG. 4. CNBr digestion of Bpa²-PTHrP-(1–36) complexes formed with hP1R or hP1R mutants substituted at methionine residues. A, gel-purified complexes formed between ^{125}I-Bpa²-PTHrP-(1–36) and hP1R (lane 1), hP1R-M425L/M441L/M445I (lane 2), hP1R-M425L (lane 3), hP1R-M441L (lane 4), or hP1R-M445I (lane 5) were treated with CNBr in 70% formic acid for 24 h, as described under “Experimental Procedures,” and the resulting digested samples were analyzed by Tricine/SDS-PAGE autoradiography, with equal amounts of radioactivity loaded in each lane. B, gel-purified complexes formed between ^{125}I-Bpa²-PTHrP-(1–36) and hP1R-M414V (lane 1), hP1R-M224L (lane 2), or hP1R-M231I (lane 3) were treated with CNBr for 24 h, and the digestion products were analyzed by Tricine/SDS-PAGE autoradiography, with equal amounts of radioactivity loaded in each lane. The positions of the cross-linked ^{125}I-Bpa²-PTHrP-(1–36) ligand was also analyzed (A, lane 6; B, lane 4). The positions of size markers (in kDa) are indicated.

FIG. 5. CNBr digest of complexes formed between Bpa²-PTHrP-(1–36) and hP1RCAM-HR or hP1R mutants substituted at methionine residues in the TM6/ECL3 region. Gel-purified complexes formed between ^{125}I-Bpa²-PTHrP-(1–36) and hP1R and hP1R-M441L/M445I (A, lanes 1 and 2), hP1RCAM-HR, and hP1RCAM-HR-M144V (B, lanes 1 and 2), or hP1RCAM-HR and hP1RCAM-HR-M414V (C, lanes 1 and 2) were treated with CNBr for 24 h, and the digested samples were then analyzed by Tricine/SDS-PAGE autoradiography, with equal amounts of radioactivity loaded in each lane. The positions of size markers (in kDa) are indicated.

FIG. 6. Binding and cAMP-signaling properties of Bpa²-PTHrP-(1–36) with hP1R and hP1R mutants. A, the effects of varying doses of Bpa²-PTHrP-(1–36) on intracellular cAMP levels in COS-7 cells expressing hP1RCAM-HR (open circles), hP1RCAM-HR-M425L (filled squares), or hP1RCAM-HR-M414V (open triangles) are shown. Asterisks indicate values that are statistically different from basal (dashed line; *, p < 0.05; **, p < 0.0001). B, the effects of varying doses of Bpa²-PTHrP-(1–36) on intracellular cAMP levels in COS-7 cells expressing hP1R (closed circles), hP1R-M425L (open squares), or hP1R-M414V (filled triangles) are shown. The asterisks indicate values that are statistically different from the response observed with hP1R at the corresponding dose (**, p < 0.0001). C, the capacity of Bpa²-PTHrP-(1–36) at varying doses to inhibit the binding of ^{125}I-bPTH-(3–34) to hP1RCAM-HR (open circles), hP1RCAM-HR-M425L (filled squares), or hP1RCAM-HR-M414V (open triangles) is shown. D, the capacity of Bpa²-PTHrP-(1–36) at varying doses to inhibit the binding of ^{125}I-bPTH-(3–34) to hP1R (closed circles), hP1R-M425L (open squares), or hP1R-M414V (filled triangles) is shown. Data shown are compiled from 3 to 6 independent replicate experiments, each performed in duplicate, as indicated in Table I.
In the presence of Met425, the cross-linking of Bpa2-PTHrP-(1–36) to site A in hP1RCAM-HR resulted in a detectable signal, whereas cross-linking with the Leu mutation at Met425 specifically altered the photoreactive environment of the ligand. Thus, the Leu mutation at Met425 in hP1RCAM-HR specifically alters the inverse agonist activity of amino-terminally intact PTHrP ligands modified with Bpa at position 2.

**Discussion**

This study was aimed at mapping the cross-linking site for a ligand determinant of inverse agonism in a constitutively active hPTH-1 receptor. Our data indicate that Bpa2-PTHrP-(1–36) can cross-link to more than one site in hP1CAM-HR, one of these sites (site A) occurs within an interval that spans the TM6/extracellular loop 3 (ECL3) boundary and is delimited by receptor residues Pro345 and Met441, and the other (site B) occurs within an adjacent interval that contains TM4 and TM5 and is delimited by residues Ala313 and Met414 (Fig. 8). Cross-linking of Bpa2-PTHrP-(1–36) to site A in hP1CAM-HR resulted in an inverse agonist activity in hP1CAM-HR specifically alters the inverse agonist activity of amino-terminally intact PTHrP ligands modified with Bpa at position 2.

**Table I**

| Receptor                  | Binding | cAMP |        |        |        |        |        |        |
|---------------------------|---------|------|--------|--------|--------|--------|--------|--------|
|                           | B<sub>max</sub> | rPTH-(1–34) | Bpa2-PTHrP-(1–36) | Basal | rPTH-(1–34) | Bpa2-PTHrP-(1–36) |
| hP1R                      |       |      |        |        |        |        |        |        |
| hP1R-M425L                | 100 ± 20 | 22 ± 3 | 32 ± 8 | (5)    | 6.5 ± 0.6 | 1.3 ± 0.5 | 385 ± 35 | 62 ± 11 | 56 ± 6 |
| hP1R-M414V                | 107 ± 26 | 23 ± 3 | 34 ± 10 | (4)    | 7.1 ± 0.8 | 1.6 ± 0.5 | 424 ± 30 | 39 ± 6 | 105 ± 6 |
| hP1R-M441I/M445I          | 86 ± 17 | 24 ± 5 | 41 ± 11 | (4)    | 6.3 ± 0.7 | 2.5 ± 0.7 | 415 ± 24 | 59 ± 24 | 65 ± 5 |
| hP1R<sub>36</sub>         | 30 ± 3  | 13 ± 2 | 22 ± 3  | (3)    | 4.7 ± 0.4 | 0.7 ± 0.2 | 284 ± 10 | 10 ± 7 | 9 ± 2  |

**FIG. 7. Effect of Met<sup>425</sup> → Leu substitution in H223R on responsiveness to amino-terminally intact or truncated inverse agonists.** COS-7 cells transiently transfected with hP1CAM-HR (A) or hP1CAM-HR-M425L (B) were treated with either buffer alone (basal) of buffer containing [Bpa<sup>2</sup>]-PTH-(1–34) (1 × 10<sup>-6</sup> M) or [Leu<sup>11</sup>,D-Trp<sup>12</sup>,Tyr<sup>36</sup>]-rPTH-(1–36) (1 × 10<sup>-6</sup> M) for 30 min at room temperature, and the resulting intracellular cAMP levels were measured, as described under “Experimental Procedures.” Asterisks indicate values that are statistically different from basal (dashed line, * p < 0.05, ** p < 0.001). Data are compiled from three independent replicate experiments, each performed in duplicate.
Inverse Agonist Cross-linking to the PTH/PTHrP Receptor

To the wild-type hP1R, cross-linking of our Bpa2-containing analog to site A in agreement with the previously reported study of Behar et al. (6), in which it was concluded that a similar Bpa2-containing PTHrP-(1–36) analog cross-linked to an overlapping interval in TM6 and that the side chain methyl group of Met425 was the principal contact site. Although in our study we could not confirm cross-linking to Met425, we did find that CNBr did not cleave at this residue, in either hP1RCAM-HR or hP1R, when Bpa2-PTHrP-(1–36) was cross-linked to site A. This result suggests that cross-linking in the A interval occurred near enough to Met425 to interfere with the CNBr cleavage reaction.

With both hP1RCAM-HR and hP1R we found abundant CNBr-dependent release of free ligand, which has been observed in other studies (35, 36) when cross-linking occurred to the side chain methyl group of a methionine residue. In our study, we were unable to verify that cross-linking occurred to the side chain methyl group of any of the methionine residues in the juxtamembrane region of the receptor, as we continued to obtain adequate cross-linking to mutant receptors altered at one or several of these methionines, and we observed abundant CNBr-dependent release of free ligand from each of the mutant receptor complexes (Figs. 3–5). Whether this free ligand originated from a cross-link to a site within the TM6 interval which gives rise to the ~5.0-kDa band (6), or to a site within another interval, could not be determined from our current data. In any case, it seems likely that this release of free ligand involves a cleavage mechanism that differs from those that have been described previously for CNBr action at methionine residues.

Within the A cross-linking interval of hP1RCAM-HR, we identified Met425 as a functional determinant of the inverse agonist activity of Bpa2-PTHrP-(1–36); mutation of this residue to leucine eliminated the inverse agonist activity that Bpa2-PTHrP-(1–36) exhibited on the constitutively active receptor and resulted instead in a weak partial agonist response, without affecting basal signaling activity. The M425L mutation in hP1RCAM-HR also eliminated the inverse agonist activity of Bpa2-PTH-(1–34), but it had no effect on the inverse agonist activity of the amino-terminally truncated analog [Leu1-D-Trp12]PTHrP (5–36) (Fig. 7). These observations are consistent with the hypothesis that Bpa2-PTHrP-(1–36) and [Leu1-D-Trp12]PTHrP (5–36) mediate inverse agonism through distinct mechanisms that involve critical contacts to different sites in the receptor (28). In the wild-type hP1R, the M425L mutation increased the agonist efficacy of Bpa2-PTHrP-(1–36), suggesting that the methionine residue at this position in the wild-type receptor plays a role in mediating the antagonist action of the ligand, as suggested previously (6). Consistent with this hypothesis, the human PTH-2 receptor has valine at the corresponding TM6 position (37) and elicits a full agonist response to Bpa2-PTHrP-(1–36) (9).

The M425L mutation in hP1RCAM-HR resulted in a reduction of Bpa2-PTHrP-(1–36) cross-linking to site A and an increase in cross-linking to site B. In the wild-type receptor the M425L mutation also resulted in a small but reproducible increase in the amount of Bpa2-PTHrP-(1–36) cross-linking to site B (Figs. 3B and 4A). The rank order of cross-linking to site B (relative to site A) seen in these receptors, hP1RCAM-HR-M425L > hP1RCAM-HR > hP1R-M425L > hP1R (Fig. 3B), correlated with the amount of receptor signaling activity that would be expected for these receptors in the presence of the low concentration (~0.5 nM) of Bpa2-PTHrP-(1–36) used in the cross-linking experiments (Fig. 6, A and B, and Table I). This raises the intriguing possibility that the benzophenone group of Bpa2-PTHrP-(1–36) contacts site A when the receptor is in an inactive conformation and contacts site B when the receptor is in an active conformation. Further investigations are needed to assess this possibility more directly.

The precise molecular mechanisms by which Bpa2-PTHrP-(1–36) mediates inverse agonism with H223R are unknown, but contact of the benzophenone adduct of the ligand to a site in or near the extracellular end of TM6 seems to be involved. In the wild-type P1R, a shift in the position of TM6 away from that of TM3 has been postulated to be a key step in the activation process (38), as suggested for other G-protein-coupled receptors as well (38, 39). In our other studies of the P1R, we have identified several point mutations in the TM6/ECL3/TM7 region (e.g. at Thr427, Trp437, Gln440, and Gln451, Fig. 1) that specifically affect the functional properties of PTH ligands modified at positions 1 and/or 2 (30, 40). Interactions between the Bpa group of Bpa2-PTHrP-(1–36) and the extracellular end of TM6 may facilitate or enable the binding of the ligand to an inactive state of hP1RCAM-HR and thus shift the equilibrium of G-protein-coupled and -uncoupled receptors in the direction of the latter (12, 13). The lack of inverse agonism exhibited by Bpa2-PTHrP-(1–36) with hP1RCAM-TP, which contains a Thr→Pro mutation at position 410 in TM6 (Fig. 1), as well as the reduced overall apparent cross-linking efficiency observed with this ligand/receptor pair, as compared with that seen with hP1RCAM-HR (28), provides evidence to suggest that the topological configuration of the heptahelical bundle of hP1RCAM-TP is different from that of hP1RCAM-HR, particularly in regard to TM6. A recent computer simulation analysis of hP1RCAM-HR and hP1RCAM-TP suggests that whereas the two mutations in these receptors produce nearly the same increased solvent accessibility of intracellular loop 3, they do so by inducing different conformational changes and motions in the TM helices (41). Recent experimental data (28, 42, 43) provide support for the notion that activate-state PTH-1 receptors can assume different conformations, and this tertiary variability may give rise to

**Fig. 8.** Two sites of Bpa2-PTHrP-(1–36) cross-linking to hP1RCAM-HR. Shown is a schematic of the amino-terminal portion of Bpa2-PTHrP-(1–36) (dark coil) in association with the heptahelical portion of hP1RCAM-HR, as viewed from the extracellular surface of the complex. As described in the text, the photoreactive benzoyl-phenylalanine side chain (dark-light shaded circle) at position 2 of the ligand was found to contact two different sites in the receptor: site A (as contacted by the dark shaded circle) occurred within the interval Pro125 to Met441, which included the TM6/ECL-3 boundary; site B (as contacted by the light shaded circle) occurred within the interval Ala113 to Met414, which included TM4 and TM5. Contact to site A correlated with the inverse agonist activity of the ligand, as the Met425→Leu mutation at the extracellular end of TM6 abolished inverse agonist activity and caused a relative shift of Bpa2-PTHrP-(1–36) cross-linking from site A to site B. The site B contact is shown to occur to TM5 because of previous mutational studies that suggest functional interactions between residues in this helical domain (Ser430 and He431) and residue 2 in the ligand (40).
altered ligand selectivity profiles (12), such as that observed with Bpa2-PTHrP(1–36) (28).

In summary, we have identified two receptor sites of contact, one in TM6 and another in the TM4/TM5 region, between a ligand determinant of inverse agonism and a constitutively active PTH-1 receptor using a photoaffinity cross-linking approach. We identified a single residue (Met425) in the TM6 contact region that, when mutated, changes the response induced by the ligand analog from that of inverse agonism to that of partial agonism, and the mutation results in a relative shift in the site of cross-linking from TM6 to the TM4/TM5 region. The results thus provide insights into the processes by which the PTH-1 receptor binds peptide ligands and isomerizes between active and inactive states.

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