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

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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,Ile⁶,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-Trp¹⁴]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 PTH1 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 (Kₐ > 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 carboxyl-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), Thr⁴¹⁰ → Pro...
 agonist at hP1RCAM-HR, as it is at the wild-type hP1R. The struc-

ture of TM6 (6). We hypothesized that mapping the cross-linking

site of Bpa2-PTH-(1–34) to the P1R consti-
tutive activity and, potentially, ligand inverse agonist activity

Thus, systems are now emerging for the study of P1R consti-
tutive activity and, potentially, ligand inverse agonist activity in

model disease states.

In cell-based settings, inverse agonist activity at both hP1R-CAM-HR and hP1R-CAM-TP has been demonstrated with the previously described antagonist peptides [Leu11,D-Trp12]hPTHrP-(7–34)-amide and [b-Trp12]hPTH-(7–34)-amide (27). Recently, we reported (28) the characterization of several new PTH and PTHrP antagonist analogs that act as inverse agonists at each of the constitutively active P1R mutant. One of these analogs, [Bpa2,ile1,Trp23,Tyr26]PTHrP-(1–36)-amide (Bpa2-PTHrP-(1–36)), contains a photobleachable amino acid derivative (p-benzoyl-L-phenyl-
alanine) at position 2 and functions as an inverse agonist at hP1R-CAM-HR; however, it is not an inverse agonist at hP1R-CAM-TP or hP1R-CAM-IR, and it is a weak partial agonist at the wild-type receptor (28). Importantly, the photobleachable position 2 amino acid derivative itself is responsible for the inverse agonist activity of this analog at hP1R-CAM-HR, as the otherwise isosteric parent peptide [ile1,Trp23,Tyr26]PTH-(1–36)-amide (valine at position 2) is an agonist at hP1R-CAM-HR as it is at the wild-type hP1R. The struc-
tural 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 ϕ-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,ile1,Arg11,Trp12]PTH-(1–36)-amide, cross-links at or near Met425, in the extracellular end of TM6 (6). We hypothesized that mapping the cross-linking site for Bpa2-PTHrP-(1–36) in hP1R-CAM-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 hP1R-CAM-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—** [ile1,Trp23,Tyr26]PTH-(1–36)-amide (PTH-(1–36)), [Bpa2,ile1,Trp23,Tyr26]PTHrP-(1–36)-amide (Bpa2-PTHrP-(1–36)), [Nle1,Trp23,Tyr26]PTH-(1–34)-amide (PTH-(1–34)), [Bpa2,Nle1,ile1,Trp12]bPTH-(1–34)-amide (bPTH-(1–34)), [ile1,Trp23,Tyr26]bovine PTH-(1–34)-amide (Bap2-PTH-(1–34)), [Nle1,Trp23,Tyr26]bovine PTH-(3–34)-amide (bPTH-(3–34)), [ile1,Leu11,b-Trp12]PTH-(5–36)-amide ([Leu11,b-Trp12]PTHrP-(5–36)) were synthesized by the Protein and Peptide Core Facility at Massachu-
setts General Hospital (Boston) by the solid-phase method on PerkinElmer Life Sciences models 430A and 431A synthesizers. Pepti-
tides were purified by reverse-phase high pressure liquid chromato-
graphy, and their compositions were confirmed by amino acid analysis and mass spectroscopy. Na13C1 (specific activity 2000 Ci/mmol) was purchased from PerkinElmer Life Sciences. Dulbecco’s modified Eagle’s medium, tryp-
in/EDTA, penicillin G/streptomycin, and horse serum were pur-
chased from Life Technologies, Inc. Fetal bovine serum and Tricine were pur-
chased from Sigma. Trifluoroacetic acid was purchased from Pierce. Cya-
ngonone bromide (CNBr) was purchased from Serva Fine Chemicals/ Boehringer Ingelheim (Heidelberg, Germany). 15C-Methylated protein

- **Cell Culture/DNA Transfection—** COS-7 cells were cultured in Dul-
becco’s modified Eagle’s medium supplemented with 10% heat-inacti-
vated 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 and cAMP assays and 6-well plates for cross-linking 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.

- **Radiolabeling of Peptides—** Radiolabeled peptides were prepared by chloramine-T iodination, followed by high pressure liquid chromatography purification using a 30–50% acetonitrile gradient in 0.1% triflu-
oroacetic acid over 30 min.

- **Radioligand-Receptor Binding and cAMP Accumulation Assays—** Binding assays were performed as described previously (31). In brief, the [125I]-labeled radioligand bPTH-(3–34) was incubated with cells expressing wild-type or mutant P1R in the presence of varying concen-
trations (0–10−10 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 previously (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 X SDS-PAGE sample buffer to give final concentrations of 4% SDS, 80 mM Tris-HCl (pH 6.8), 20% glycerol, 0.2% bromophenol blue, and 100 mM dithiothreitol.

- **SDS-PAGE Analysis/Purification—** The samples in SDS-PAGE sam-
ple buffer were incubated at room temperature for 2 h and then sub-
jected to SDS-PAGE analysis (10% acrylamide) performed according to the method of Laemmli (32). For visualization of the intact cross-linking complexes, gels were dried and exposed 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 electrodissociation in a dialysis bag (molecular mass cut-off = 12,000 Da) at 100 V for 2 h. Eluted samples were concentrated using Centricon-10 tubes (Millipore Co., 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 mM) in 70% formic acid at 20 °C for 24 h. After digestion, CNBr and formic acid were removed by repetitive lyophilization. Samples were suspended in SDS-

PAGE sample buffer, incubated at room temperature for 2 h, and then analyzed by Tricine/SDS-PAGE (12% acrylamide) performed according to the method of Schägger and von Jagow (33). Dried gels were sub-
jected to autoradiography at −80 °C. For all studies shown, similar results were obtained in at least three separate experiments.

- **Data Calculation—** Calculations were performed using Microsoft Ex-
cel. Nonlinear regression analyses of binding and cAMP dose-response data were performed using the four-parameter equation: \( y = Min + ((Max \ - \ Min)/(1 + ([C]/IC_{50}^{max}))) \). 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 radiolabeled 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.
plexes 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 PTHrP 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 Met⁴²⁵ because mutation of this residue to leucine markedly reduced cross-linking efficiency. In our current study, introduction of the Met⁴²⁵ → 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_HR, this same mutation resulted in a comparable or if not greater yield of cross-linking product, relative to that obtained with the control hP1R_CAM_HR (Fig. 3A, lanes 1 and 2). Upon CNBr digestion, both hP1R-M425L and hP1R_CAM_HR-M425L complexes yielded a prominent −3.5-kDa band (Fig. 3B). These results imply that within both hP1R and hP1R_CAM_HR 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 digests of both the hP1R_CAM_HR and hP1R_CAM_HR-M425L complexes (Fig. 3A, lanes 1–3). Upon CNBr digestion, both hP1R_CAM_HR and hP1R_CAM_HR-M425L complexes yielded a prominent −5.0-kDa band (Fig. 3B). These results imply that within both hP1R and hP1R_CAM_HR 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 digests of both the hP1R_CAM_HR and hP1R_CAM_HR-M425L 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_HR 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
FIG. 2. Cross-linking of Bpa²-PTHrP-(1–36) to hP1RCAM-HR and hP1R. A, Bpa²-PTHrP-(1–36) was bound to COS-7 cells transiently transfected with hP1RCAM-HR (lane 1) or hP1R (lane 2) or pCDNA vector alone (lane 3), and photoaffinity cross-linking was induced, as described under “Experimental Procedures.” Cells were lysed, and equal volumes of the resulting lysates were analyzed on SDS-PAGE followed by autoradiography at −80 °C. The positions of size markers (in kDa) are indicated. B, gel-purified complexes formed between ¹²⁵I-Bpa²-PTHrP-(1–36) and hP1RCAM-HR (lanes 1 and 2) or hP1R (lanes 3 and 4) were treated with 70% formic acid in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of CNBr for 24 h. The samples were analyzed by Tricine/SDS-PAGE autoradiography, with equal amounts of radioactivity loaded into each lane. The positions of size markers (in kDa) are indicated; the 2.35- and 3.5-kDa size markers are not resolved.

FIG. 3. Cross-linking of Bpa²-PTHrP-(1–36) to hP1RCAM-HR, hP1R, and hP1R-M425L. A, hP1RCAM-HR-M425L (lane 1), hP1R (lane 2), or hP1R-M425L (lane 4) were treated with 70% formic acid in the absence (lane 1) or presence (lanes 2 and 4) of CNBr for 24 h. The samples were analyzed by Tricine/SDS-PAGE autoradiography, with equal amounts of radioactivity loaded in each lane.

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ligand cross-linked to the Pro⁴¹⁵-Met⁴⁹⁹ 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 Met⁴¹¹ → Leu/Met⁴⁴⁵ → 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 appeared. This new band correlated with the size predicted for Bpa²-PTHrP-(1–36) cross-linked to the Pro⁴¹⁵-Met⁴⁹⁹ fragment (Fig. 5A, lanes 1 and 2). These results thus established the identity of the ~5.0 kDa obtained with the wild-type hP1R as Bpa²-PTHrP-(1–36) cross-linked to the receptor fragment Pro⁴¹⁵-Met⁴⁹⁹.

To determine the origin of the ~5.0-kDa band in hP1RCAM-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 Bpa²-PTHrP-(1–36) and hP1RCAM-HR-M441V, 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 correlated with the size predicted for Bpa²-PTHrP-(1–36) cross-linked to the receptor fragment Ala³¹³-Met⁴⁴⁴ (Fig. 1). Consistent with this interpretation, the ~5.0-kDa band was no longer observed in the CNBr digestion of the complex formed with hP1RCAM-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 hP1RCAM-HR corresponds to Bpa²-PTHrP-(1–36) cross-linked to the receptor fragment Pro⁴¹⁵-Met⁴⁹⁹.

The size of the ~17-kDa band observed in the CNBr digests of hP1RCAM-HR was most consistent with Bpa²-PTHrP-(1–36) cross-linked to the receptor segment Ala³¹³-Met⁴⁴⁴ (Fig. 1). The apparent shift of this ~17 to ~18 kDa seen in the digest of the hP1RCAM-HR-M441V mutant receptor described above (Fig. 5B, lanes 1 and 2) verified this assignment, as it confirmed that Met⁴⁴⁴ was a boundary residue for the cross-linked receptor interval. The overall mapping results obtained with hP1RCAM-HR therefore suggest that Bpa²-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 Pro⁴¹⁵-Met⁴⁴⁴ and gives rise to the ~5.0-kDa band, and the other (site B) is delimited by residues...
Ala313–Met414 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 (Ala313–Met441) but instead produced this band together with an 18-kDa band (Ala313–Met425; Fig. 5B) indicated that CNBr could cleave at Met425 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 Met425 that we observed when Bpa2-PTHrP-(1–36) was cross-linked to site A (e.g. within the Pro415–Met414 segment) suggested that cross-linking occurred at or near this residue. Moreover, mutation of Met425 to Leu in hP1R CAM-HR resulted in a reduction of 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
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positional and 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.

Table I

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Binding</th>
<th>cAMP</th>
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<tbody>
<tr>
<td></td>
<td>B&lt;sub&gt;max&lt;/sub&gt;</td>
<td>r&lt;sub&gt;PTh-(1–34)&lt;/sub&gt;</td>
</tr>
<tr>
<td>hP1R</td>
<td>100 ± 20</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>hP1R-M425L</td>
<td>107 ± 26</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>hP1R-M414V</td>
<td>86 ± 17</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>hP1R-M441I/M445I</td>
<td>30 ± 3</td>
<td>13 ± 2</td>
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% hP1R<sub>Cam-HR</sub> | 100 ± 15 | 6.1 ± 1.2 | 9.2 ± 1.4 | 4 (4) | 66 ± 4 | 0.9 ± 0.1 | 147 ± 13 | — | 73 ± 4 | (6) |
| hP1R<sub>Cam-HR</sub>M425L | 94 ± 5  | 4.1 ± 0.4 | 6.3 ± 1.3 | 3 (3) | 62 ± 3 | 0.5 ± 0.1 | 121 ± 7 | — | 113 ± 5 | (5) |
| hP1R<sub>Cam-HR</sub>M414V | 49 ± 9  | 2.8 ± 0.5 | 3.8 ± 0.5 | 3 (3) | 81 ± 2  | 1.2 ± 0.3 | 136 ± 7 | — | 82 ± 4 | (5) |
| hP1R<sub>Cam-HR</sub>M441I/M445I | 13 ± 2 | 1.6 ± 0.1 | 2.8 ± 0.4 | 3 (3) | 21 ± 1 | 0.6 ± 0.1 | 54 ± 4 | — | 58 ± 4 | (3) |

position 425 can alter the photoreceptor environment of the benzophenone adduct of the bound ligand. We therefore investigated if mutation of Met<sup>425</sup> would affect the functional response of the receptor to Bpa<sup>2</sup>-PTHrP-(1–36). As shown in Fig. 6A, the M425L mutation in hP1R<sub>Cam-HR</sub> eliminated the inverse agonist response to Bpa<sup>2</sup>-PTHrP-(1–36) and instead resulted in a weak partial agonist response. This effect occurred without a change in the level of constitutive signaling activity (basal cAMP = 62 ± 3 and 66 ± 5 pmol/well for hP1R<sub>Cam-HR</sub>M425L and hP1R<sub>Cam-HR</sub>-M425L, respectively, p = 0.4), nor a change in Bpa<sup>2</sup>-PTHrP-(1–36) binding affinity (IC<sub>50</sub> values = 6.3 ± 1.3 and 9.2 ± 1.4 nM, respectively, p = 0.1; Table I and Fig. 6C). In the hP1R, the M425L mutation increased the agonist efficacy of Bpa<sup>2</sup>-PTHrP-(1–36) (Fig. 6B), again without affecting basal signaling or binding affinity (Fig. 6D and Table I). In contrast, the M414V mutation, analyzed as a control, had little or no effect on the signaling or binding properties of Bpa<sup>2</sup>-PTHrP-(1–36) in either hP1R<sub>Cam-HR</sub> or hP1R (Fig. 6, A–D). The binding and signaling properties of rPTH-(1–34) were not affected by either the M425L or the M414V mutation (Table I).

We also examined whether or not the M425L mutation would affect the signaling activity of a Bpa<sup>2</sup>-containing PTH analog, [Bpa<sup>2</sup>Nle<sup>6</sup>,Ile<sup>8</sup>,NaL<sup>23</sup>Arg<sup>13</sup>Ser<sup>26</sup>,Trp<sup>37</sup>Tyr<sup>34</sup>bovine PTH-(1–34)-amide (Bpa<sup>2</sup>-PTH-(1–34)), which has been reported to function as a fully efficacious but reduced potency agonist at the wild-type hP1R (36). As with Bpa<sup>2</sup>-PTHrP-(1–36), this analog functioned as an inverse agonist at hP1R<sub>Cam-HR</sub> and this inverse agonist activity was abolished by the M425L mutation (Fig. 7, A and B). In contrast to the effects on inverse agonist activity of the Bpa<sup>2</sup>-containing PTH and PTHrP analogs, the M425L mutation had no effect on the inverse agonist activity of [Leu<sup>11</sup>]-bPTHrP (5–36), which lacks position 2 altogether (Figs. 7, A and B). Thus, the Leu mutation at Met<sup>425</sup> in hP1R<sub>Cam-HR</sub> specifically alters the inverse agonist activity of amino-terminally intact PTH or 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 Bpa<sup>2</sup>-PTHrP-(1–36) can cross-link to more than one site in hP1R<sub>Cam-HR</sub>—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 Pro<sup>445</sup> and Met<sup>441</sup>, and the other (site B) occurs within an adjacent interval that contains TM4 and TM5 and is delimited by residues Ala<sup>413</sup> and Met<sup>414</sup> (Fig. 8). Cross-linking of Bpa<sup>2</sup>-PTHrP-(1–36) to site A in hP1R<sub>Cam-HR</sub> resulted in an ~5.0-kDa CNBr-generated band, whereas cross-linking to site B resulted in an ~17-kDa CNBr-generated band (Fig. 2B, lane 2). Cross-linking to site A was also detected in the wild-type hP1R, but there was little or no evidence for cross-linking to site B in this receptor (Figs. 2B and 3B). With respect
the wild-type hP1R, cross-linking of our Bpa2-containing analog to site A is 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 site 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 site 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, with-
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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REFERENCES


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