Molecular Approximation between a Residue in the Amino-terminal Region of Calcitonin and the Third Extracellular Loop of the Class B G Protein-coupled Calcitonin Receptor*

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The calcitonin receptor is a member of the class B family of G protein-coupled receptors, which contains numerous potentially important drug targets. Delineation of themes for agonist binding and activation of these receptors will facilitate the rational design of receptor-active drugs. We reported previously that a photolabile residue within the carboxyl-terminal half (residue 26) and mid-region (residue 16) of calcitonin covalently label the extracellular amino-terminal domain of this receptor (Dong, M., Pinon, D. I., Cox, R. F., and Miller, L. J. (2004) J. Biol. Chem. 279, 1167–1175). Chimeric receptor studies support the importance of this region and suggest important contributions of extracellular loop domains. To examine whether other parts of the ligand may contact those loops, we developed another probe that has its photolabile site of labeling within the amino-terminal half in position 8 of the ligand. This probe was a full agonist (EC$_{50}$ = 565 ± 67 pM), stimulating cAMP accumulation in receptor-bearing human embryonic kidney 293 cells in a concentration-dependent manner. It bound specifically and saturably (K$_{d}$ = 14.3 ± 1.9 nM) and was able to efficiently label the calcitonin receptor. By purification, specific cleavage, and sequencing of labeled wild-type and mutant calcitonin receptors, the site of attachment was identified as residue Leu$_{368}$ within the third extracellular loop of the receptor, a domain distinct from that labeled by previous probes. These data are consistent with a common ligand binding mechanism for receptors in this important family.

Calcitonin (CT), secreted by the thyroid gland in response to elevations in blood calcium levels, is a peptide hormone that regulates calcium by inhibition of osteoclast-mediated bone resorption (1, 2). CT acts on bone and kidney to maintain calcium homeostasis and is also present in the central nervous system, where it has anorectic and analgesic effects (3). It has calcium homeostasis and is also present in the central nervous system, where it has anorectic and analgesic effects (3). It has

CT is a relatively large peptide that contains 32 amino acids and has a diffuse pharmacophoric domain. Although residues throughout the entire length have been demonstrated to be critical for its biological activity, the amino-terminal residues of CT contain key determinants for its receptor agonist selectivity (1, 2). Truncation of the first seven amino-terminal residues that includes a disulfide bond between residues 1 and 7 results in antagonist action (4, 5). Residues 8 through 22 tend to form an amphiphilic α-helical structure that is important for high affinity binding (1).

CT exhibits its agonist activities through binding to the CT receptor, a member of the class B family of guanine nucleotide-binding protein (G protein)-coupled receptors that have the seven-transmembrane-domain structure. Although they have topology similar to that of class A receptors, members of class B family share less than 12% amino acid identity with the more extensively studied receptors in the class A family. Class B receptors have distinct signature sequences, including a long complex amino-terminal domain with six conserved cysteine residues that are believed to be involved in intradomain disulfide bonds critical for establishing functional receptor conformation (6–10). Members included in this family are receptors for moderately large peptides having diffuse pharmacophoric domains, such as secretin, calcitonin, glucagon, vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating polypeptide, and parathyroid hormone, sharing 30 to 50% homology with each other.

The unique amino-terminal domain of the CT receptor has been shown to be critical for agonist binding and receptor activation using chimeric receptor studies (11–13). This represents a consistent theme for other class B family members (14–19). Photoaffinity labeling is a more direct approach for exploration of spatial approximations between residues within a ligand and within its receptor. Using this approach, we have recently demonstrated that probes incorporated a photolabile p-benzoyl-i-phenylalanine (Bpa) residue in the carboxyl-terminal half and mid-region of the human CT peptide ligand, in positions 16 and 26, covalently label receptor residues Phe$_{137}$ and Thr$_{190}$, respectively. Both are within the extracellular amino terminus of the CT receptor, with the former adjacent to the first transmembrane segment and the latter within the distal amino-terminal tail of the receptor (20). Pham et al. (21) recently reported that the ligand binding domain for a photolabile probe incorporating a Bpa in position 19 was localized to a segment (Cys$_{134}$-Lys$_{141}$) within the receptor amino terminus that included residue Phe$_{137}$, a site labeled by our position 16 probe (20). All these data consistently support a critical role of the amino terminus of the CT receptor in ligand binding.

However, chimeric receptor studies have also suggested that the extracellular loop domains of the CT receptor play a role
complementary to the amino terminus in agonist binding and receptor activation (13), but no such regions of the CT receptor have yet been shown to be spatially approximated with the ligand using photoaffinity labeling. To determine whether other portions of CT might contact these loop domains, we developed another probe in which its photolabile residue is closer to the amino terminus of the ligand, in position 8. This probe (Bpa8 probe), was designed to incorporate a photolabile Bpa within its amino-terminal half, and characterized as a fully efficacious agonist that bound to its receptor saturably and specifically and that efficiently covalently labeled the CT receptor in a single distinct domain. This domain was identified as the third extracellular loop of the CT receptor by chemical cleavage of labeled wild-type and mutant receptors, and the labeled residue was identified as Leu<sup>688</sup> using radiochemical Edman degradation sequencing. This site is distinct from sites of labeling with all previous probes (20, 21). This new pair of residue-residue approximation sites was generated in which Leu<sup>368</sup> was mutated to Ala (L368A), and Tyr at position 12, acting as a site of radioiodination. This probe was synthesized by manual solid-phase techniques and purified to homogeneity up to seven cycles, in a manner that has been previously reported (22). The Bpa<sup>8</sup> probe was designed to incorporate a photoaffinity-bearing membranes from HEK-CTR cells were incubated with 0.1 nm<sup>125</sup>I-(Bpa<sup>8</sup>)human calcitonin-32 in the presence of increasing concentrations (0–1 μM) of the Bpa<sup>8</sup> probe, CT, or other peptide ligands, for 1 h at room temperature in Krebs-Ringer-HEPES medium containing 0.01% soybean trypsin inhibitor and 0.2% bovine serum albumin. Bound and free radioligand were separated by centrifugation and washing, with bound radioactivity quantified in a γ-spectrometer. Nonspecific binding was determined in the presence of 1 μM CT and represented <20% of total binding. The same assay was also used to characterize the binding activity of the new receptor mutants transiently expressed on COS cells. Binding curves were analyzed and plotted using the nonlinear regression analysis routine for radioligand binding in the Prism software package (GraphPad Software, San Diego, CA). Binding kinetics was determined by analysis with the LIGAND program of Munson and Rodbard (31).

### EXPERIMENTAL PROCEDURES

### Materials—
Human CT, human parathyroid hormone, and human glucagon-like peptide-1 were purchased from Bachem (Torrance, CA). Cyanogen bromide (CNBr), solid phase oxidant N-chloro-benezensulfonyl fluoride and m-maleimidobenzoyl-N-hydroxysulfosuccinimide were purchased from Pierce. 3-Isobutyl-1-methylxanthine and endoglycosidase F were prepared in our laboratory (25, 26). All other reagents were analytical grade.

### Peptides—
The Bpa<sup>8</sup> probe was designed to incorporate a photolabile Bpa into position 8 for covalent labeling of its receptor. This position has been demonstrated to be well tolerated for replacement by an Ile residue (20). The Bpa<sup>8</sup> probe contained a naturally occurring Tyr at position 12, acting as a site of radioiodination. This probe was synthesized by manual solid-phase techniques and purified to homogeneity by reversed-phase high performance liquid chromatography to yield specific radioactivity of 2,000 Ci/mmol (27).

### Receptor Sources—
The human embryonic kidney 293 cell line stably expressing the human CT receptor isoform II (HEK-CTR) that we used previously (20) were again used as the source of receptors for the current studies. Cells were cultured at 37 °C in a 5% CO<sub>2</sub> environment on Falcon tissue culture plasticware in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal cote-2 (Hyclone Laboratories, Logan, UT). They were passaged twice a week and lifted mechanically before use. In this work, it was necessary to develop a new CT receptor mutant that eliminated a naturally occurring site for CNBr cleavage in the third extracellular loop of the receptor. This represented Met<sup>787</sup> to Leu (M787L) receptor construct. In addition, another CT receptor construct was generated in which Leu<sup>688</sup> was mutated to Ala (L688A), representing mutation of the site of labeling by the Bpa<sup>8</sup> probe. Both mutants were prepared using an oligonucleotide-directed approach with the QuickChange site-directed mutagenesis kit from Stratagene, with the products verified by direct DNA sequencing (28). Both were expressed transiently on COS cells (American Type Culture Collection, Manassas, VA) after transfection using a modification of the DEAE-dextran method (15). Cells were harvested mechanically 72 h after transfection. Plasma membranes were prepared from the above receptor-expressing cells using methods reported previously (29).

### Biological Activity Assay—
This was performed by measuring the intracellular cAMP accumulation in HEK-CTR cells in response to stimulation by CT or the Bpa<sup>8</sup> probe, using a competitive-binding assay (Diagnostic Products Corporation, Los Angeles, CA). In brief, cells grown in 24-well plates were stimulated by increasing concentrations (0–1 μM) of CT or the Bpa<sup>8</sup> probe for 30 min at 37 °C in Krebs-Ringer-HEPES medium (25 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, and 2 mM CaCl<sub>2</sub>) containing 1 mM 3-isobutyl-1-methylxanthine, 0.01% soybean trypsin inhibitor, 0.1% bacitracin, and 0.2% bovine serum albumin. The incubation was stopped by adding ice-cold 6% perchloric acid. After adjusting the pH to 6 with KOH solutions, cell lysates were cleared by centrifugation at 3,000 rpm for 10 min, and the supernatants were used in the assay, as described previously (30). Radioactivity was quantified by scintillation counting in a Beckman Coulter LS6000 liquid scintillation counter. Assays were performed in duplicate and repeated in at least three independent experiments. This assay was also used to functionally characterize the new receptor mutants expressed transiently on COS cells.

### RESULTS

### Probe Characterization—
The Bpa<sup>8</sup> probe was designed to incorporate a photolabile Bpa within its amino-terminal half, in the position of Met<sup>8</sup> (Fig. 1). It was synthesized by manual solid phase techniques and purified by reverse-phase HPLC. It was characterized to demonstrate the expected molecular mass by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. As shown in Fig. 2, this probe bound saturably and specifically to CT receptor-bearing HEK-CTR cells,
Ligand-binding Domain of Calcitonin Receptor

Fig. 1. Peptides. Shown are the sequences of the natural human CT and the photolabile Bpa probe. This probe incorporated a Bpa into position Met for covalent labeling of the CT receptor. The peptide was radiiodinated oxidatively on the Tyr residue in position 12.

Fig. 2. Characterization of the Bpa probe. Left, abilities of increasing concentrations of natural CT or the Bpa probe or other related peptides, human parathyroid hormone (PTH, secretin (Sec) and glucagon-like peptide-1 (GLP-1), to compete for binding of the radioligand, 125I-human CT-32, to HEK-CTR cells. Values are calculated as percentages of maximal saturable binding observed in the absence of competitor. They are expressed as means ± S.E. of duplicate data from three independent experiments. Right, both natural CT and the Bpa probe stimulated intracellular cAMP accumulations in HEK-CTR cells in a concentration-dependent manner. Values are expressed as the means ± S.E. of at least three independent experiments.

Although it had affinity lower than natural CT (K<sub>i</sub> values: Bpa probe, 14.3 ± 1.9 nM; CT, 1.1 ± 0.1 nM; B<sub>max</sub>, 103,000 ± 10,000 binding sites/cell), as expected, other distinct peptides in the calcitonin family had not competed for binding using concentrations as high as 1 μM (Fig. 2). The Bpa probe was a full agonist, stimulating intracellular cAMP accumulation in HEK-CTR cells in a concentration-dependent manner (basal, 27 ± 5 pmoL/million cells; maximal, 235 ± 5 pmoL/million cells), although with lower efficacy than natural CT (Bpa probe, EC<sub>50</sub>, 563 ± 67 pM; CT, EC<sub>50</sub>, 26 ± 6 pM; Fig. 2).

Photoaffinity labeling of the CT receptor—The Bpa probe was next used to explore its ability to covalently label the CT receptor. As shown in Fig. 3, this probe covalently labeled the CT receptor saturably and specifically, and the labeling was inhibited by increasing concentrations of natural CT (IC<sub>50</sub> = 2.1 ± 0.4 nM) but not by other related peptides, such as parathyroid hormone, secretin, and glucagon-like peptide-1 at concentrations as high as 1 μM (Fig. 3). Consistent with our previous work on labeling of this receptor with the Bpa<sup>6</sup> and Bpa<sup>26</sup> probes, the labeled receptor migrated at approximate M<sub>r</sub> 97,000 and shifted to M<sub>r</sub> 52,000 after deglycosylation. This band was not present in samples prepared from non-receptor-bearing parental HEK cell membranes. The high efficiency for covalent labeling of the CT receptor with this probe provided the opportunity for the identification of the specific site of receptor labeling by this probe.

Active Site Identification—To gain an initial indication of receptor domains of labeling with the Bpa probe, we first chose to cleave the labeled receptor by CNBr that we have used successfully for the secretin (22, 26, 35–39) and CT (20) receptors in our laboratory. CNBr cleaves at the carboxyl side of Met residues within a protein, and its cleavage of the CT receptor would theoretically yield 15 fragments ranging from 0.1 to 11 kDa, two of which contain potential glycosylation sites. As shown in Fig. 4, CNBr cleavage of the CT receptor labeled with the Bpa probe resulted in a single band migrating at approximate M<sub>r</sub> 4,500 that migrated distinctly from the radioiodinated free probe (M<sub>r</sub> 3,500). Furthermore, deglycosylation using endoglycosidase F did not change the migration of the fragment, indicating the labeled fragment was not glycosylated. Considering the molecular mass of the attached probe (3,538 Da), the fragments Asp<sup>49</sup>-Met<sup>59</sup> at the amino terminus and Leu<sup>368</sup>-Met<sup>376</sup> within the third extracellular loop domain of the receptor (Fig. 4, highlighted in black circles) were felt to represent the best candidates for the domain of labeling with the Bpa probe.

To determine which of these domains included the site of covalent attachment, a construct was generated in which Met<sup>376</sup> was changed to Leu (M376L) to eliminate a site for CNBr cleavage. This receptor construct was expressed transiently on COS cells and functionally characterized in binding and cAMP assays. Data in Fig. 5 demonstrate that specific and high affinity binding (K<sub>i</sub> = 0.96 ± 0.08 nM; 43,000 ± 8,000 binding sites/cell) as well as stimulation of cAMP accumulation (EC<sub>50</sub> = 37 ± 5.1 pM) in response to CT in these cells were not significantly different from that in control cells expressing the
wild-type receptor (binding, $K_r = 1.1 \pm 0.06$ nM; 64,000 ± 6,000 binding sites/cell. cAMP stimulation, $EC_{50} = 26 \pm 3.3$ pM). This construct was also affinity-labeled saturably and specifically with the Bpa8 probe, with the labeled band migrating on an SDS-polyacrylamide electrophoresis gel at approximate $Mr = 97,000$ and shifting to $Mr = 52,000$ after deglycosylation (Fig. 6). CNBr cleavage of the labeled intact receptor yielded a fragment migrating at approximate $Mr = 4,500$ that was not affected by treatment with endoglycosidase F (EF). This band migrated slightly slower than the free Bpa8 probe ($Mr = 3,500$). Highlighted in bold circles in the diagram at left are two candidate fragments that best represent the receptor domain of labeling for the Bpa8 probe.

Characterization of CT Receptor Site Mutant—The L368A CT receptor construct was transiently expressed on COS cells and characterized for CT binding and CT-stimulated cAMP accumulation in these cells following the methods described in Fig. 2. Values are expressed as means ± S.E. of duplicate data from three independent experiments. WT, wild-type CT receptor; M376L, M376L mutant CT receptor.

and was studied for impact on the binding and biological activities of CT. Like that of receptor residues labeled by the Bpa16 and Bpa36 probes (20), mutation of the Leu368 residue that was covalently labeled by the Bpa8 probe had little negative effect on binding ($K_r = 1.5 \pm 0.2$ nM; 40,000 ± 6,000 binding sites/cell) and biological (EC$_{50} = 166 \pm 33$ pM) activities. This was done to confirm that there is adequate space between the relevant residues in CT and its receptor to accommodate the relatively large Bpa residue, so as not to significantly interfere with normal binding and activation.

DISCUSSION

Understanding of the molecular basis of ligand binding to a receptor can provide information useful for the rational design of receptor-active drugs. The CT receptor represents a very
important drug target, and CT has found widespread clinical use for the treatment of bone disorders since its discovery (1, 2). However, the molecular mechanism of ligand binding of CT to its receptor is not well understood. The amino terminus of the CT receptor has been shown to be critical in ligand binding by mutagenesis (11–13) and recently by photoaffinity labeling (20, 21). The current report continues to use the photoaffinity labeling approach and shows that a amino-terminal photolabile CT agonist probe labels a receptor residue (Leu368) in the third extracellular loop domain, a region distinct from the region that had been labeled by previous calcitonin probes (20, 21).

Using intrinsic photoaffinity labeling, we have previously demonstrated that both Bpa16 and Bpa26 probes labeled residues within the extracellular amino-terminal domain of the CT receptor; the former labeled a residue near the first transmembrane segment (Phe137) and the latter labeled a residue within the distal amino terminus of the receptor (Thr209) (20). However, receptor mutagenesis studies have not only suggested the importance of the amino-terminal domain of the CT receptor but also supported a complementary role for extracellular loop domains (13). In an attempt to determine whether CT might be directly interacting with such regions of the receptor body, we sought to extend our previous photoaffinity labeling studies. In the current work, we focused on the amino-terminal half of CT, a region not previously studied by photoaffinity labeling. CT contains a disulfide bond between residue Cys1 and Cys7, and this bridge is critical for its full agonist activity. Met8 is located right after this disulfide bond and it is not a conserved residue. Furthermore, substitution of this residue can eliminate the only naturally occurring site for CNBr cleavage in the ligand to prevent its cleavage during peptide mapping of the labeled receptor. It is interesting that Met8 is a position at which a predicted α-helical structure (residues 8–22) starts, as demonstrated by NMR analysis of natural CT (1). The importance of conformational flexibility around residue 8 has also been suggested (40). It is predicted by analysis of chimeras that the α-helix spanning residue 8 through 22 of CT interacts with the extracellular loop regions of the receptor (13). For all these reasons, we chose to incorporate a photolabile Bpa at the Met8 of CT, within a region that is important for high affinity binding.

In this work, we have shown that the replacement of Met8 by a Bpa had significant negative impact on both receptor binding and biological activities (Fig. 2). Although this confirms the critical importance of this residue in human CT, it is different from the Ile-substitution data, which show little negative impact on binding or biological activities (20), probably because of the smaller size of Ile than the Bpa molecule. Suva et al. have demonstrated that incorporation of an (ε-p-benzyloibenzoyl)lysine residue into Val8 of salmon CT can be tolerated without significant loss of binding and biological activities (41), indicating that the importance of this position may vary from species to species, or indicating that the substituted (ε-p-benzyloibenzoyllysine residue in this position may be better tolerated than a Bpa. Although the Bpa8 probe has lower affinity than natural CT, it bound to its receptor saturably and specifically. It was a full agonist, although with lower potency than natural CT (Fig. 2). More importantly, this probe was able to covalently label the CT receptor as efficiently (Fig. 3) as did the Bpa16 and Bpa26 probes that we used previously, demonstrating its ability to be used in exploration of the ligand-binding sites using the photoaffinity-labeling approach. As expected, this intrinsic photoaffinity-labeling approach identified a residue (Leu368) within the third extracellular loop domain of the CT receptor as the site of covalent attachment to the Bpa8 probe, a region distinct from the amino-terminal domain of the CT receptor that was labeled by the Bpa16, Bpa19, and Bpa26 probes (20, 21).

It is noteworthy that the third extracellular loop domain of the CT receptor was identified as the domain of labeling of the Bpa8 probe, because the extracellular loop regions have been shown to be critical for ligand binding by analysis of calcitonin-glucon receptor chimeras (13). The importance of the extra- cellular loop domains in ligand binding has been consistent for other members in the class B G protein-coupled receptor family, including receptors for secretin (14, 15), vasoactive intesti- nal polypeptide (42, 43), parathyroid hormone (44–46), glucagon (47, 48), growth hormone-releasing hormone (49), corticotropin-releasing factor (50), luteinizing hormone/cho- riogonadotropin (51–54), follicle-stimulating hormone (55–57), and gonadotropin-releasing hormone (58). In particular, the third extracellular loop has been shown to be important for high affinity binding for the parathyroid receptor (44, 46). This domain has also been shown to interact directly with the hor- mone ligand for another family of G protein-coupled receptors (i.e. receptors for follicle-stimulating hormone (56, 57), luteinizing hormone/choriogonadotropin (52), and gonadotropin-re-leasing hormone (58)) to activate these receptors. It is noteworthy that the third extracellular loop of G protein-coupled receptors is near the sixth transmembrane domain, which has been shown to interact directly with the amino terminus of the peptide ligand for receptors for parathyroid hormone (23, 24) and secretin (22).

It is worth of mentioning that the carboxyl-terminal residue Bpa26 of CT ligand labeled a residue within the distal amino terminus of the receptor (20), the mid-region residues Bpa16 (20) and Bpa19 (21) labeled receptor residues within the amino terminus near the first transmembrane domain, and the amino-terminal residue Bpa8 labeled a residue within the third extracellular loop of the receptor (the current work). These data suggest that when docking, the natural CT ligand is sited between two major docking domains (i.e. the amino terminus and the third extracellular loop domain). As such, it could function as a tethering ligand that could exert tension and thereby change the conformation of the body of the receptor that could be transmitted to the cytosolic face of the receptor where the G protein interaction occurs (59). A similar theme has been proposed for parathyroid hormone (60) and secretin (22) receptors, two other members of the same family of G protein-coupled receptors. Most recently, we have shown that the first four or five secretin amino-terminal residues were positioned close to the third extracellular loop of the receptor in our secretin-bound receptor model, with His1 of secretin positioned at the top of transmembrane helix six (38). Although this suggests a common mechanism for binding and activation of class B family G protein-coupled receptors, there has been a
similar proposal for members of another family, such as receptors for follicle-stimulating hormone (56, 57), luteinizing hormone/choriogonadotropin (52), and gonadotropin-releasing hormone (58).

In summary, we have now generated a new CT probe that incorporated a photolabile residue into the amino-terminal half of the ligand and have used it to explore the residue-residue approximation as docked to the CT receptor. It labeled a residue in the third extracellular loop of the CT receptor, a region that is distinct from that labeled by calcitonin probes used previously (20, 21). Thus, we have provided an additional critical spatial constraint that is useful for the modeling of the agonist-bound CT receptor. This insight supports a common mechanism for activation of class B G protein-coupled receptors involving a tethering ligand sandwiched between two critical ligand-binding domains of the receptor. This should become clearer as we add more experimentally derived constraints and develop a credible model of the agonist-bound CT receptor.

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