Design, Synthesis and Utility of Novel Benzophenone-Containing Calcitonin Analogs for Photoaffinity Labeling the Calcitonin Receptor

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ABSTRACT

Calcitonin (CT) is a 32-amino-acid calciotropic peptide hormone which acts on target cells via a G-protein-coupled seven-transmembrane receptor (CTR). In this study, we report the design, synthesis and characterization of four potent bioactive and photoreactive CT analogs, each of which contains a single benzophenone moiety inserted at different and discrete locations within the CT molecule. Replacement of all Lys residues in salmon CT (sCT) with Arg, followed by replacement of hydrophobic residues with a Lys(p-phenylbenzoyl) residue [Lys(p-phenylbenzoyl)] was found to preserve high biological activity. We substituted Val at Lys8, Leu16 and Leu19 by Lys, and acylated the N-terminus by a pBz2 moiety, thus distributing the photoaffinity moiety in the different analogs across a large portion of the CT sequence. With both transfected and endogenous CTRs from several species, all four benzophenone-containing analogs were shown to be virtually indistinguishable from the parent sCT analog in both receptor binding properties and stimulation of cAMP accumulation. Upon photolysis, in the presence of CTR, the radiiodinated photoreactive CT analog ([Arg11,18,Lys19(p-phenylbenzoyl)]sCT (K19)) covalently labels a membrane component of approximately 70 kDa. Receptor cross-linking is inhibited specifically in the presence of excess sCT. We also examined the interaction of these CT analogs with a hemagglutinin (HA) epitope-tagged CTR. The HA-CTR displayed CT binding and CT-dependent cAMP stimulation identical with native CTR. Both K19 and another bioactive analog ([Arg11,18,Lys8(p-phenylbenzoyl)]sCT (K8)) specifically photoaffinity cross-link to the HA-CTR. These benzophenone-containing CT analogs should facilitate studies of hormone-receptor interactions and allow the direct identification of a CT binding domain(s) within the receptor by the analysis of photochemically cross-linked conjugates.

Calcitonin is a potent, clinically useful inhibitor of bone resorption (Civitelli et al., 1988; Gruber et al., 1984; Mazzuoli et al., 1986; Reginster et al., 1987), which exerts its effects on target cells in (osteoclasts) via specific CTRs (Chambers and Dunn, 1983). Exposure of osteoclasts to CT rapidly results in the retraction of podosomes and the “ruffled border” membrane (Holtrup et al., 1974; Chambers and Magnus, 1982), an activation of adenylyl cyclase (Murad et al., 1970) and a decrease in bone resorption (Chambers et al., 1985). Among the CTRs of several species, the homology is greatest at the disulfide-bridged N-terminus. Furthermore, all known CT sequences have Gly and Pro at the C-terminus (Findlay et al., 1985). Binding of CT to CTRs results in the activation of not only Gs, but also G-proteins involved in activation of phospholipase C, protein kinase C and intracellular inositol trisphosphate and calcium levels (Chakraborty et al., 1991; Stroop et al., 1993; Stroop and Moore, 1994; Livesey et al., 1984; Findlay et al., 1980; Lin et al., 1991).

Multiple isoforms of the CTR from several different species have been cloned and expressed (Lin et al., 1991; Gorn et al., 1992; Sexton et al., 1993; Albrandt et al., 1993; Yamin et al., 1994). Sequence comparison reveals that the CTR belongs to a subgroup of the superfamily of G protein-coupled receptors composed of seven putative transmembrane helical domains.

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ABBREVIATIONS: BIN67, human ovarian cell line; Boc, t-butoxycarbonyl; CT, calcitonin; CTR, calcitonin receptor; COS-7, receptor negative monkey kidney cell line; DCM, dichloromethane; DMF, N,N-dimethylformamide; DEMEM, Dulbecco’s modified minimum essential medium; e, eel; p, porcine; h, human; PBS, fetal bovine serum; Fmoc, 9-fluorenylmethoxycarbonyl; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; HF, hydrogen fluoride; IBMX, 3-isobutyl-1-methyuxanthine; K8, [Arg11,18,Lys19(p-phenylbenzoyl)]sCT; K16, [Arg11,18,Lys15(p-phenylbenzoyl)]sCT; K19, [Arg11,18,Lys19(e-phenylbenzoyl)]sCT; LLC-PK1, porcine kidney cell line; Nα, [Arg11,18,Nα-pBz2]sCT; pMBHA, p-methylbenzhydrylamine resin; PBS, phosphate-buffered saline; PTH, parathyroid hormone; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

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separated by exofacial and intracellular loops. This group includes the PTH/PTH-related protein, PTH2, secretin, vasoactive intestinal peptide, glucagon-like peptide 1, growth hormone-releasing hormone and glucagon receptors (Segre and Goldring, 1993 and references therein; Usdin et al., 1995). The homology within this subgroup is 30 to 60%, but CTR has less than 12% sequence identity with other G protein-coupled receptors outside the subgroup (Segre and Goldring, 1993). The structural features common to all members of this subgroup are: a relatively long amino-terminal extracellular domain which has multiple potential N-glycosylation sites, and a highly conserved pattern of cysteine residues in the amino-terminal domain and the first and second exofacial loops.

All CT analogs prepared and characterized to date have been designed without benefit of knowledge regarding the structural features of the CTR, nor details of the nature of the bimolecular interaction between hormone and receptor. With the availability of cloned CTRs comes an opportunity to pursue a new approach for the design of improved CT analogs based on directly mapping the bimolecular hormone-receptor interface. The conventional approach for studying ligand binding and activation of G protein-coupled receptors is the analysis of transiently expressed mutant and/or chimeric receptors (Segre and Goldring, 1993; Bergwitz et al., 1996). Although informative, this approach assumes that structural manipulation of the receptor does not result in conformational changes either at the modified site or at a site(s) distant from the modification. In contrast, our studies use the stable expression of native receptor and specific radiolabeled, photoreactive benzophenone-containing, bioactive CT analogs which cross-link irreversibly and specifically at the ligand binding site(s) within the CTR. Our approach should enable the unambiguous identification of a CTR domain(s) which is in direct contact with the ligand.

In this study, we report the design and synthesis of a set of four novel photoreactive benzophenone-containing sCT analogs and their in vitro biological characterization, with CTRs from three different species. In addition, we also describe specific photo-cross-linking of the porcine CTR by use of two of these analogs, analog IV (K19) (see fig. 1 for schematic structure) and analog II (K8). This study describes our efforts to “photoaffinity scan” (Williams and Shoelson, 1993) the CT-CTR bimolecular interface. Elucidation of the molecular details of the hormone-receptor interaction may provide important new insights into the mechanism of ligand recognition and CT-mediated signal transduction and possibly aid in the development of novel, rationally designed CT-based therapeutics.

**Methods**

**Materials.** Na-Boc-protected amino acids, TFA, diisopropyldiamine, N,N-dicyclohexylcarbodiimide, N-hydroxybenzotriazole and the pMBHA resin (1% cross-linked, 0.77 mmol of nitrogen/g) were purchased from Applied Biosystems, Inc. (Foster City, CA). DCM, DMF and methanol, all B and J brand, were obtained from Baxter (McGraw Park, IL). Triethylamine was purchased from Fisher Scientific (Springfield, NJ). TFA (spectrophotometric grade), acetic anhydride, ethyl acetate, anisole, p-benzoyl benzoic acid, petroleum ether and anhydroxy ether were purchased from Aldrich Chemicals (Milwaukee, WI). HF was purchased from Matheson (Seacacus, NJ). Iodogen was purchased from Pierce Chemical Co. (Rockford, IL). [Des-Cys1,Asu3] eel CT (Ectalbin) ([Asu1,7]eCT) was purchased from Bachem (Torrance, CA). RPMI 1640, Ca++, Mg++-free Hanks’ balanced salt solution was purchased from Life Technologies (Grand Island, NY). Tissue culture disposables and plasticware were obtained from Corning (Corning, NY). All tissue culture media, FBS and I-glutamine were purchased from Gibco-BRL (Gaithersburg, MD). Adenosine and IBMX were obtained from Research Biochemicals Inc. (Natick, MA). Adenine, cAMP, ADP and ATP were purchased from Sigma Chemical (St. Louis, MO). Na125I (2025 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL).

**General peptide synthesis and purification.** The synthesis of Na-Boc-Lys(Np-benzoylbenzoyl)-OH (Boc-Lys(Np-bz2)-OH) was reported previously (Nakamoto et al., 1995). All peptides were synthesized on a 430A Automated Peptide Synthesizer (Applied Biosystems Inc., Foster City, CA) with version 1.2 software of the dicyclohexylcarbodiimide/N-hydroxybenzotriazole cycles. Details of the recoupling and capping cycles and unique synthetic steps used in the preparation of the various analogs are described under the specific entries. The following side-chain-protected Na-Boc-amino acid derivatives were used in the course of the automated solid-phase peptide synthesis: Arg(N2-Tosyl), Cys(Sp-MeBzl), Glu(OBzl), His(N9-Bom), Lys(N9-2Cl-Z), Lys(N9-Fmoc), Ser(OBzl), Thr(OBzl) and Tyr(2-Br-Z).

Cleavage of the peptide from the pMBHA resin with concomitant removal of side-chain-protecting groups was achieved by treatment with liquid HF in the presence of 10% anisole (20 ml/g resin-bound peptide) for 1 h at 0°C. The mixture of crude peptide and resin obtained after removal of HF under vacuum was washed consecutively with petroleum ether and anhydroxy ether. The dry mixture of crude peptide and resin was extracted with 50% (v/v) acetic acid (100 ml) and water (500 ml). The solution of the crude peptide was filtered through a sintered glass funnel. The oxidation of the crude peptide in the filtrate was carried out by dropwise addition of sulfuric acid, 1% in water, to reach the total concentration of 0.5% (v/v) in anhydrous acid to a magnetically stirred solution. The crude peptide was purified on a RP-HPLC equipped with a PrePack cartridge, Vydac C18, 300Å 15- to 20-μm column. The eluting solvents used were: A, 0.1% (v/v) TFA in H2O; and B, 0.1% (v/v) TFA in acetonitrile. The linear gradient used consisted of 0 to 100% (v/v) B for 10 min followed by 10 to 50% (v/v) B for the next 190 min at a flow rate of 70 ml/min and monitored at 220 nm. The fractions from the preparative RP-HPLC were analyzed on a Vydac C18 protein column (0.54 x 15 cm, 5 μm) with a linear gradient from 0% to 95% of B in A during 30 min at a flow rate of 1.5 ml/min and monitored at 220 nm. Fractions containing the pure peptide were pooled, concentrated under reduced pressure and lyophilized. The detailed analytical data are reported in table 1.

**Synthesis of Na[pBz2][Arg11,18]sCT (I).** The synthesis of Boc[Arg18]sCT(17-32)-pMBHA resin was carried out on a 0.5-mmol
scale. The protocol consisted of double couplings followed by capping with Ac₂O for the following positions: Pro⁳⁰, Thr⁳¹, Asn²⁶-His²⁷. At this stage the resin-bound peptide was split into two halves and the synthesis continued with double couplings on a 0.25-mmol scale. The free α-amino terminus of [Arg¹¹,¹₈]sCT was blocked with p-benzoyl benzoic acid (2 mmol, 0.45 g) converted in situ to the corresponding anhydride. Purification of the crude peptide was carried out on a RP-HPLC equipped with a PrePack cartridge (Vydac C18, 300 Å, 15–20 μm). The linear gradient used consisted of 0 to 40% (v/v) B in A in 200 min at a flow rate of 70 ml/min, monitored at 220 nm.

**Synthesis of [Arg¹¹,¹₈]Lys⁸(p-Bz₂)₂sCT (II, K₅).** The synthesis of Boc[Arg¹¹,¹₈]Lys⁸(p-Bz₂)₂sCT was carried out as described above for analog I with use of the other 0.25 mmol of protected resin-bound peptide from above. At position 8, Boc-Lys(p-Bz₂)₂-OH (2 mmol, 0.909 g) was incorporated by a single extended (overnight) symmetrical anhydride coupling cycle followed by capping with Ac₂O. The rest of the synthesis followed the protocol as described for analog I. The linear gradient used in the preparative RP-HPLC purification consisted of 0 to 50% (v/v) B in A over 200 min.

**Synthesis of [Arg¹¹,¹₈]Lys⁸(p-Bz₂)₃sCT (III, K₁₆).** The synthesis of Boc[Arg¹¹,¹₈]Lys⁸(p-Bz₂)₃sCT MBHA resin was carried out as described for I. Deprotection with TFA/DCM of 0.25 mmol of resin-bound peptide was followed by neutralization with disopropylethyl amine. Coupling with Boc-Lys(ε-Fmoc)-OH (2 mmol, 0.94 g) was carried out by the symmetrical anhydride method, as described previously (Nakamoto et al., 1995; Chorev et al., 1991). Cleavage of the ε-Fmoc was achieved by 20% piperidine in DMF (1 × 1 min, followed by 1 × 1 min). The resin was washed consecutively with DMF (1 × 1 min), DCM (4 × 1 min) and DMF (2 × 1 min). Coupling of p-benzoyl benzoic acid (2 mmol, 0.45 g) to the free α-amino group of the resin-bound peptide was carried out as a standard symmetrical anhydride coupling cycle. The rest of the synthesis follows the same procedure described for analog I. RP-HPLC purification was carried out as described above for analog II.

**Synthesis of [Arg¹¹,¹₈]Lys⁸(p-Bz₂)₄sCT (IV, K₁₉).** The synthesis of Boc-εCT(20–32)-pMBHA resin was carried out on a 0.25-mmol scale by use of single couplings followed by capping with Ac₂O. Incorporation of Boc-Lys(ε-Fmoc)-OH and replacement of the Nε-Fmoc protecting group with pBz₂ group followed the procedure described above for analog III. Extension of the Boc[Lys⁸(p-Bz₂)₄sCT]pMBHA included double coupling cycles followed by capping with Ac₂O at the following positions: Arg¹¹-Leu¹⁶, Arg¹¹ and Val¹⁰-Ser¹. RP-HPLC purification was carried out as described for analog II.

**Radioiodination of [Asu¹,¹₇]εCT and [Arg¹¹,¹₈]εCT analogs K₅ and K₁₉.** Solutions of analogs (1 μg/ml) were prepared by dissolving the peptide in 10 mM acetic acid and diluting with twice the volume of 100 mM sodium phosphate buffer, pH 7.4. The peptide solution (80 μl) was added to Na¹²⁵I (10 μl, 1 mCi) placed into a borosilicate tube coated with Iodogen (5 μg). Radioiodination was carried out at room temperature for 2 min and the reaction terminated by the addition of aqueous 0.1% TFA (300 μl). The entire reaction mixture was purified on a RP-HPLC NovaPak C18 (3.9 × 150 mm) (Waters, Milford, MA) with a solvent system of A, 0.1% TFA in H₂O, B, 0.1% TFA in acetonitrile. Linear gradient of B in A; B = 5 to 95% in 30 min.

**Cell culture and transfection.** The porcine kidney proximal tubule cell line LLC-PK₁ (Goldring et al., 1978) and COS-7 (Gorn et al., 1992) cells were maintained in DMEM supplemented with 10% FBS. Human ovarian cells (BIN67) endogenously expressing hCTR (Gorn et al., 1992) were maintained in 60% DMEM/20% Ham’s F-12/20% FBS. Stably transfected HEK-293 cells, expressing rCTR isoform C1a (Sexton et al., 1993), were maintained in DMEM, supplemented with 5% FBS and 80 μg/ml G418 (Sigma, St. Louis, MO). Transient transfection of pCTR into COS-7 cells was performed with 1 to 5 μg of the various CTR constructs with either calcium phosphate or DEAE-dextran as described previously (Suva et al., 1991).

**HA-pCTR mutagenesis.** The cloning of the cDNA encoding the pCTR has been reported (Lin et al., 1991). A human influenza virus HA-tagged pCTR (HA-pCTR) was prepared by site-directed mutagenesis of the wild-type pCTR cDNA (Bergwitz et al., 1996). Insertion of the nucleotide sequence CCTTACGATGTTCCGGATTACGCT into the structure of the HA-pCTR on the cell surface was obtained by ¹²⁵I-[Asu¹,¹₇]εCT binding and by HA antibody (Berkley Antibodies, Berkley, CA) binding performed as described (Gardella et al., 1996; Bergwitz et al., 1996). Transiently transfected COS-7 cells were rinsed with binding buffer (50 mM Tris-HCl, pH 7.7; 100 mM NaCl; 5 mM KCl; 2 mM CaCl₂, 5% heated-inactivated FBS). Binding buffer (250 μl) containing HA monoclonal antibody (1.5 μg/ml) was added and incubated for 2 h at 15°C. The labeled cells were rinsed with binding buffer and 250 μl of binding buffer containing ¹²⁵I-labeled goat anti-mouse IgG was added and incubated for 2 h at 15°C. The cells were then washed again with binding buffer and lysed with 5 M NaOH (0.5 ml) and the entire cell lysate counted in a gamma counter (Packard, Cobra Auto-Gamma 5000, model 5002, Meriden, CT).

**Receptor binding assay.** Cells were grown in 10-cm² tissue culture dishes, harvested with 0.05% (w/v) trypsin, counted in a Coulter counter (Coulter Electronics, Hialeah, FL) and resuspended at approximately 10⁶ cells/0.2 ml in binding buffer (11 mM glucose in PBS). The determination of specific CTR binding was performed as described previously (Lin et al., 1991; Gorn et al., 1992, Hourassi et al., 1994). Cells were incubated with ¹²⁵I-labeled [Asu¹,¹₇]εCT or pBz₂-containing CT analogs (100,000 cpm/10 μl), with or without increasing concentrations of nonradioactive εCT in binding buffer.
overnight at 4°C. Cells were resuspended and a 100-μl aliquot added to 200 μl of cold (4°C) 10% sucrose. The mixture was centrifuged at 10,000 × g for 5 min at 4°C and the supernatant carefully removed by vacuum. The cell pellet was then counted in a gamma counter.

**cAMP radioimmunoassay.** Cells in 24-well tissue-culture plates were incubated with various CT analogs for 10 min in growth media in the presence of 1 mM IBMX. The incubation was terminated by the addition of perchloric acid (final concentration, 30%) and the samples neutralized with potassium bicarbonate, acetylated, and the total cAMP (medium + cells) determined by radioimmunoassay as described previously (Pines et al., 1994). Radioactivity was counted in a scintillation counter. Curves were fitted by CA Cricket Graph III v 1.0 (Computer Associates, Islandia, NY).

**Photoaffinity labeling of CTRs.** Cells were harvested and resuspended (∼1–2 × 10^6/200 μl) in 0.5 ml PBS. On ice, 200 μl of the cell suspension was aliquoted into Fisher’s glass Wasserman tubes (Pittsburgh, PA). Cell suspensions were incubated with 1 nM ([1–2 × 10^6 cpn/ml] iodinated photolabile K19 or K8 analog for 30 min on ice. Specific competition of the photoaffinity labeling was performed by a 15-min preincubation of 5 μg sCT with the cell suspension. The tubes were then irradiated on ice for 15 min with a focused 365 nm Blak-Ray 75 Watt UV lamp: model B100A (San Gabriel, CA), placed approximately 10 cm above the ice bath. After photolysis, the cell suspensions were centrifuged (4°C, 2000 × g) for 10 min, and the cell pellets were washed twice with ice-cold PBS. The final cell pellets were resuspended in 250 μl Laemmli sample buffer containing 15% β-mercaptoethanol and boiled for 5 min. Samples were stored at −20°C until analysis by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The presence or absence of a collection of protease inhibitors (bacitracin, leupeptin, trasylol) did not affect the binding or photolysis experiments (data not shown). Similarly, polyacrylamide gel electrophoresis analysis in the presence or absence of 15% β-mercaptoethanol showed no observable differences in the size or specificity of the photolabeled bands (data not shown).

**Results**

**Synthesis and characterization.** The syntheses used three different modalities for introduction of the βBz₂ moiety into resin-bound peptide: 1) For No (pBz₂) [Arg^{11,18}]sCT (I), at the end of polypeptide chain assembly, the Nα-terminal Boc-protecting group was removed and the free amino end group was blocked by in situ generated p-benzoyl benzoic anhydride. 2) A similar approach was used to prepare [Arg^{11,18}, Lys^{16}]sCT and [Arg^{11,18}, Lys^{19}]sCT (II, and IV, K19, respectively). After extension of the resin-bound peptide by the orthogonally protected Lys residue Boc-Lys(Fmoc)-OH, the Fmoc side-chain-protecting group was removed and the free ε-amino function acylated by in situ generated p-benzoyl benzoic anhydride. 3) Preformed Boc-Lys(p-Bz₂)-OH was coupled onto the resin-bound peptide. Synthesis was then continued with use of the solid-phase peptide synthesis method. With this strategy we were able to prepare all the βBz₂-containing analogs in porcine LLC-PK1 cells. (A) Competitive inhibition of \( {^{125}}I \)-[Asu²]-sCT binding to LLC-PK1 cells. Values for cpm/100,000 cells are mean ± S.E.M. of triplicate samples. Results represent at least three additional experiments. (B) sCT- and pBz₂-containing analog-stimulated cAMP accumulation. Results represent three additional experiments. Values for cAMP (pmol/100,000 cells) are mean ± S.E. of triplicate samples. Results represent at least three additional experiments.

**Binding and cAMP accumulation.** The characterization of the binding and stimulation of CAMP responsiveness of the pBz₂-substituted CTs was carried out in several cell lines expressing CTRs from different species: porcine kidney cells (LLC-PK1), expressing wild-type pCTR (Goldring et al., 1978); human ovarian tumor cells (BIN 67) expressing wild-type hCTR (Gorn et al., 1992); COS-7 cells transiently transfected with the HA-pCTR or wild-type pCTR; and human embryonic kidney cells (HEK 293) stably expressing the rat CTR isof orm C1a (Sexton et al., 1993). The rank order of potencies of the pBz₂-substituted CTs was very similar in all CTR-expressing systems examined (see figs. 2 through 7). Except for No(pBz₂) [Arg^{11,18}]sCT (I), analogs II to IV were similar to sCT in terms of biological properties. The avidity of analog I for CTR in BIN 67 and HA-pCTR was approximately one order of magnitude lower than that observed for sCT (see fig. 2A). The affinity of analog I for CTR in BIN 67 and HA-pCTR was approximately one order of magnitude lower than that observed for sCT (see figs. 4A and 5A, respectively) and only 2- to 3-fold lower in HEK-293/C1a cells (see fig. 3A). The efficacy of these analogs in the various CTR systems corresponded well with their specific receptor affinity (Quiza et al., 1997). Analog I was one order of magnitude less potent than sCT in stimulating cAMP accumulation (see figs. 2B, 3B and 5B). We selected K19 ([Arg^{11,18}, Lys^{16}(p-Bz₂)]sCT (IV)) as a representative pBz₂-substituted CT analog to carry out photo-induced cross-linking studies. Competition binding experiments carried out with K19 and sCT in the presence of radioiodinated-

![Fig. 2. Biological activity of sCT- and pBz₂-containing analogs in porcine LLC-PK1 cells.](image-url)
K19 (\(^{125}\text{I}-\text{K19}\)) revealed identical binding curves with an IC\(_{50}\) of \(~7\) nM (fig. 6A). In addition, the stimulation of cAMP accumulation by sCT and pBz\(_2\)-substituted analogs in COS-7 cells transiently transfected with either the wild-type pCTR or the HA-pCTR were virtually identical (fig. 6B). Apparently, tagging the N-terminus of the pCTR with the HA epitope did not modify its functional behavior.

**Photoaffinity cross-linking studies.** Photoaffinity cross-linking of \(^{125}\text{I}-\text{K19}\) to LLC-PK1 cells revealed a single radiolabeled band of approximately 70 kDa (fig. 7, lanes 1 and 2) which was competed by incubation in the presence of \(10^{-6}\) M sCT. In the absence of UV irradiation, no cross-linking was observed (fig. 7, lanes 3 and 4). Similarly, we observed efficient and specific cross-linking of \(^{125}\text{I}-\text{K19}\) to COS-7 cells transiently transfected with pCTR, yielding a single radiolabeled 70 kDa band similar to the one observed with the wild-type pCTR in LLC-PK1 cells (fig. 8). A similar approximately 70 kDa band was also observed after cross-linking to either human ovarian cells BIN-67 or HEK-293/ C1a cells (data not shown).

**Discussion**

We took advantage of a previous report which demonstrated that replacement of all Lys residues by Arg renders a fully active CT analog, [Arg\(^{11,18}\)]sCT (D’Santos et al., 1988). In addition, numerous studies point to the importance of the amphiphilicity of the CT sequence for full bioactivity (Segre et al., 1987; Rittel et al., 1983, 1985; Moe and Kaiser, 1985; Green et al., 1988; Epand et al., 1983, 1985; Moe and Kaiser, 1985; Green et al., 1987; Rittel et al., 1976). Therefore, we chose to introduce the pBz\(_2\) moiety as a side-chain modification of a Lys residue (fig. 1), replacing an endogenous hydrophobic amino acid residue in the native CT sequence. The advantages of this approach are: 1) it is predicted to preserve the essential amphipathic nature of the CT sequence and 2) it allows selective postsynthetic manipulation of a purified N-terminal protected CT analog containing a single Lys residue which provides a single amino group for postsynthetic modification. The four CT analogs were prepared in an effort to investigate the bimolecular interaction between CT and its receptor by use of a benzophenone-based photoaffinity labeling approach (Dormán and Prestwich, 1994; Williams and Shoelson, 1993, Zhou et al., 1997).

**Fig. 3.** Biological activity of sCT- and pBz\(_2\)-containing analogs in stably transfected HEK-293 cells expressing the rat C1a CTR isoform. (A) Competitive inhibition of \(^{125}\text{I}-\text{Asu}^{1,7}\)eel-CT binding to C1a cells by sCT and pBz\(_2\)-containing analogs. Values for cpm/well are mean \pm S.E.M. of triplicate samples. Results represent at least two additional experiments. (B) sCT- and pBz\(_2\)-containing analogs-stimulated cAMP accumulation. Results represent three additional experiments. Values for cpm/100,000 cells are mean S.E.M., \(\pm N\) a.

**Fig. 4.** Competitive inhibition of \(^{125}\text{I}-\text{Asu}^{1,7}\)eel-CT binding to BIN 67 human ovarian cells by sCT- and pBz\(_2\)-containing analogs. Values for cpm/100,000 cells are mean \pm S.E.M. of triplicate samples. Results are representative of at least two additional experiments. sCT; \(\bullet\); No(pBz\(_2\))[Arg\(^{11,18}\)]sCT (I); \(\bigcirc\); [Lys\(^{16}\)(e-pBz\(_2\))[Arg\(^{11,18}\)]sCT (II); \(\Delta\); [Lys\(^{16}\)(e-pBz\(_2\))[Arg\(^{11,18}\)]sCT (III); \(\bigcirc\); [Lys\(^{16}\)(e-pBz\(_2\))[Arg\(^{11,18}\)]sCT (IV).

Competitive binding to the HA-pCTR and stimulation of cAMP accumulation by the four pBz\(_2\)-containing CT analogs I to IV, were very similar to those observed for the other cell lines expressing CTR (figs. 2–6). Based on these pharmacological criteria, N-terminal HA-tagging of the pCTR preserves full receptor functionality; specific photoaffinity cross-linking of either \(^{125}\text{I}-\text{K19}\) or \(^{125}\text{I}-\text{K8}\) to the HA-pCTR transiently expressed in COS-7 cells was observed. Both photoaffinity ligands labeled a specific membrane component of approximately 70 kDa (fig. 8). The apparent electrophoretic mobility of the HA-pCTR was slightly retarded (fig. 8). The decreased mobility of the photolabeled HA-pCTR was presumably caused by some modification of receptor conformation and/or post-translational modifications such as glycosylation, which did not affect receptor function (fig. 6). Similar observations of altered receptor mobility have been made with other epitope-tagged receptors (Emrich et al., 1993).

**Fig. 2.** Biological activity of sCT- and pBz\(_2\)-containing analogs in stably transfected HEK-293 cells expressing the rat C1a CTR isoform. (A) Competitive inhibition of \(^{125}\text{I}-\text{Asu}^{1,7}\)eel-CT binding to C1a cells by sCT and pBz\(_2\)-containing analogs. Values for cpm/well are mean \pm S.E.M. of triplicate samples. Results represent at least two additional experiments. (B) sCT- and pBz\(_2\)-containing analogs-stimulated cAMP accumulation. Results represent three additional experiments. Values for cpm/100,000 cells are mean \pm S.E.M., \(\pm N\) a.
Rittel and co-workers (1976) reported that elimination of the \(\alpha\)-amino function from the N-terminus of CT or its acet-ylation, results in enhancement of hypercalcemic potency relative to the parent peptide. Apparently, an increase in hydrophobicity at the N-terminus caused by acylation of N\(\alpha\) with \(p\)Bz2 (\(I\)) does not maintain the same level of CT-like agonist activity. The preservation of affinities and efficacy, similar to those of sCT, in analogs \(II\) to \(IV\) is essential for their utility in photoaffinity labeling studies probing ligand-receptor interactions.

In this report we demonstrate the use of two photoactive CT analogs (K19 and K8) to specifically cross-link the expressed pCTR. The photoaffinity cross-linking of the pCTR was achieved with high specificity. Cross-linking of either K19 or K8 to the endogenous or transiently transfected pCTR identified a single \(\sim 70\) kDa radiolabeled band (fig. 8). Previous studies of cross-linking to the rat CTR report inconsistent results with sodium dodecyl sulfate gel estimates of both molecular size and the number of cross-linked bands (D’Santos \(et\) \(al\)., 1988). D’Santos and co-workers (1988) identified two radiolabeled bands of 71 and 88 kDa in rat osteoclasts and a single 88 kDa band in rat UMR106–06 osteosarcoma cells. The differences between these studies and our current work may relate to species differences (rat vs. porcine) and/or to different degrees of receptor glycosylation (Quiza \(et\) \(al\)., 1997). More recently, Quiza and colleagues (1997) reported chemical cross-linking to transiently transfected pCTR with an apparent molecular weight of 57 kDa. Cross-linking to porcine hypothalamic membranes, endogenously expressing pCTR revealed a specific band of \(\sim 69\) kDa (Quiza \(et\) \(al\)., 1997), similar to our observations in LLC-PK1 cells (fig. 7). However, we note that in our photaffinity labeling studies of the human CTR expressed in BIN67 cells and the transfected rat C1a CTR, a single radiolabeled band of approximately 70 kDa is observed (data not shown). Previous studies of the CTR (D’Santos \(et\) \(al\)., 1988) and the PTH/PTH-related protein receptor (Karpf \(et\) \(al\)., 1987; Goldring \(et\) \(al\)., 1984) belonging to the same subfamily of seven transmembrane G protein-coupled receptors have used chemical cross-linking techniques which are less selective than the photoaffinity method used herein. In all our photofinity cross-linking experiments, a specifically labeled CT-CTR complex is observed with a molecular weight of \(\sim 70\) kDa.
Taking account a molecular weight of approximately 3.5 kDa for $^{125}$I-K19 or $^{125}$I-K8, the mass of the expressed pCTR (either endogenous or transfected) must be approximately 66 kDa, which is larger than the molecular weight predicted from translation of the cDNA sequence of pCTR (Lin et al., 1991). This size difference is presumably the result of post-translational modifications of the CTR, such as glycosylation. The high degree of the photo-cross-linked complex in both LLC-PK1 cells and HA-pCTR transfected cells suggests that these cells may be useful for more detailed mapping of CT-CTR interactions.

In the absence of direct physical methods to obtain the details of the tertiary structure of either the native CTR or the bound conformation of CT, the only practical method for mapping ligand-receptor interactions unambiguously is a direct one, based on the identification and analysis of specific submolecular ligand-receptor cross-linking domains and sites. The photoaffinity-based method we are pursuing offers the potential to obtain information regarding the hormone-receptor interface by identifying the site(s) of contact between CT and the CTR. The approach permits the isolation of a covalent hormone-receptor complex, which can then be subjected to exhaustive specific chemical and/or enzymatic cleavage to generate a cross-linked hormone-receptor domain. The successful application of this strategy and the availability of additional CT photoaffinity analogs should permit the unambiguous identification of a hormone-receptor contact domain(s) and ultimately specific amino acid-to-amino acid contact points.

Specific tagging of CTR with HA may be instrumental in this approach. Immunoprecipitation of a HA-pCTR-CT conjugate by commercially available anti-HA-antibodies could obviate the need for high-affinity antibodies to the CTR and could become an essential step in the purification scheme toward the identification of a CT-CTR contact domain. The premise on which the epitope-tagging technique is based and which is fully applicable to our system are (Geli et al., 1988): (1) the epitope-tagged CTR should be localized at the authentic cellular compartment in the transfected cell; (2) the epitope-tagged CTR should retain full biological function; and (3) the HA-pCTR should cross-link specifically with the benzophenone-containing sCT analogs described here. The data presented in this report demonstrate the fulfillment of these requirements. In addition, several successful applications of an epitope-tagging strategy, in general, and the HA-epitope tag in protein purification schemes, in particular, have been reported (Field et al., 1988; Qian et al., 1993; Czech et al., 1993; Chen et al., 1993; Bergwitz et al., 1996).

Photoaffinity scanning (Williams and Shoelson, 1993) of the CTR and the identification of CT-CTR contact domains, and eventually specific contact points, should provide direct insight into the nature of the interaction between CT and its receptor, which may further our understanding of hormone binding and subsequent signal transduction in the CT system. Future efforts may suggest new directions for the rational design of novel analogs of smaller size and perhaps reduced antigenicity and tachyphylactic potential, which could improve CT therapy for osteoporosis, Paget’s disease, hypercalcemia and other disorders of bone and mineral metabolism. The studies described in this report provide, for the first time, evidence that the CT-CTR interaction may be a useful target for the rational design of novel analogs.
time, the basis from which to begin an effort to directly to the CT-CTR bimolecular interface.

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References


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