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Synthesis and Characterization of Novel Biotinylated Carboxyl-terminal Parathyroid Hormone Peptides that Specifically Crosslink to the CPTH-receptor

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Abstract

Parathyroid hormone (PTH) regulates calcium, phosphorous and skeletal homeostasis via interaction with the G protein-coupled PTH/PTHrP receptor, which is fully activated by the amino-terminal 34 amino-acid portion of the hormone. Recent evidence points to the existence of another class of receptors for PTH that recognize the carboxyl (C)-terminal region of intact PTH(1–84) (CPTHRs) and are highly expressed by osteocytes. Here we report the synthesis and characterization of two novel bifunctional CPTH ligands that include benzoylphenylalanine (Bpa) substitutions near their amino-termini and carboxyl-terminal biotin moieties, as well as a tyrosine^{34} substitution to enable radioiodination. These peptides are shown to bind to CPTHRs with affinity similar to that of PTH (1–84) and to be specifically and covalently cross-linked to CPTHRs upon exposure to ultraviolet light. Crosslinking to osteocytes or osteoblastic cells generates complexes of 80kDa and 220kDa, of which the larger form represents an aggregate that can be resolved into the 80kDa. The crosslinked products can be further purified using immunoaffinity and avidin-based affinity procedures. While the molecular structure of the CPTHR(s) remains undefined, these bifunctional ligands represent powerful new tools for use in isolating and characterizing CPTHR protein(s).

Keywords

Parathyroid Hormone; Carboxy-terminal Parathyroid Hormone; Parathyroid Hormone Receptor Type-1; Benzoylphenylalanine

1. INTRODUCTION

The classical action of parathyroid hormone (PTH) is to regulate mineral ion homeostasis and bone turnover via activation of the type-1 PTH/PTHrelated peptide receptor (PTH1R), a G-protein coupled receptor that recognizes and is fully activated by the amino(N)-terminal 34 amino acid sequence of the intact hormone[1,16,17]. During the past several years, compelling evidence has emerged indicating the existence in bone cells of an additional receptor for PTH that specifically recognizes the carboxyl-(C) terminal region of the hormone (for review, see [20]). Characterization of the determinants of PTH required for high-affinity binding to this C-terminal PTH receptor (CPTHR) has identified three discrete domains within the intact hormone[7]. These domains comprise the tripeptide sequence Arg^{25}-Lys^{26}-Lys^{27}, the dibasic sequence Lys^{53}-Lys^{54} and three additional residues within the PTH(55–84) region, Asn^{57}, Lys^{65} and Lys^{72}, each of which is highly conserved across mammalian species[20]. Mutational

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analysis of these residues demonstrated a strong correlation between binding and biological functions [7].

Crosslinking is a powerful approach to covalently link a polypeptide hormone to its receptor for structural analysis [6,10]. It has been previously demonstrated that the introduction of a modified photoreactive amino acid (benzoylphenylalanine; Bpa) into the PTH sequence can lead to the identification of contact points between the ligand and the classical PTH1R [2,5, 12,13,24]. Additionally, biotinylated derivatives of polypeptide hormones have been used for analyzing the interaction between hormones and receptors in living cells and for purifying hormone-receptor complexes with affinity columns [19]. Here we report the synthesis and characterization of two novel bifunctional synthetic CPTH peptides containing Bpa substitutions in their N-terminal regions and C-terminal biotin residues. These two peptides, based upon the hPTH(23–84) and hPTH(24–84) native sequences, were long enough to include all amino-acids required for high affinity binding to the CPTHR[7] and incorporated three substitutions: a Bpa substitution (at position 24 or 26, respectively), a tyrosine at position 34 (for radioiodination) and a biotin at the C-terminus of the molecule, to allow avidin-based purification and/or detection.

Initial characterization of these two peptides revealed that they are functionally identical to the native hormone and that they can be covalently crosslinked to the CPTHR. Two-step purification demonstrated that these peptides can bind to the CPTHR upon photoaffinity crosslinking and can be used as tools to purify the hormone-receptor complex.

2. MATERIALS AND METHODS

2.1 Materials

Human [Tyr^{34}]hPTH(19–84), hPTH(53–84), [Bpa^{24},Tyr^{34}]hPTH(23–84)-biotin, [Bpa^{26},Tyr^{34}]hPTH(24–84)-biotin, [Tyr^{34}]hPTH(23–84)-biotin and [Tyr^{34}]h PTH(24–84)-biotin were synthesized by the Protein and Peptide Core Facility at Massachusetts General Hospital (Boston, MA) by solid phase method on Perkin-Elmer model 430A and 431A synthesizers. For simplicity, the following abbreviations for the CPTH analogs will be used throughout the manuscript: [Bpa^{24}, Tyr^{34}]hPTH(23–84)-biotin = Bpa^{24}-hPTH(23–84); [Bpa^{26},Tyr^{34}] hPTH(24–84)-biotin = Bpa^{26}-hPTH(24–84); [Tyr^{34}]hPTH(19–84) = hPTH(19–84);[Tyr^{34}] hPTH(23–84)-biotin = hPTH(23–84) and [Tyr^{34}]hPTH(24–84)-biotin = hPTH (24–84). Peptides were purified by reverse-phase high performance liquid chromatography, and their compositions were confirmed by amino-acid analysis and mass spectrometry. Recombinant hPTH(1–84) was a gift of NPS Pharmaceuticals. ^{125}NaI (specific activity 2000 Ci/mmol) was purchased from Perkin-Elmer Life Science. Alpha-modified Eagle's medium (α-MEM), Dulbecco's modified Eagle's medium (DMEM), Ham's F-12, penicillin G/ streptomycin (PS), trypsin/EDTA, fetal bovine serum (FBS, lot # 1147184) and horse serum were purchased from Invitrogen Life Technologies, Inc. Trifluoroacetic acid was purchased from Pierce. C^{14}-methylated protein molecular marker was purchased from *Amersham Pharmacia Biotech, and Biomax MS film was purchased from Eastman Kodak Co. All the other chemicals and reagents were from Sigma or Fisher Scientific.

2.2 Cell culture

The clonal osteocytic cell line OC59, was isolated by enzymatic digestion from calvarial bones of 18.5 day-old tsA58(+)/PTH1receptor(−/−) fetuses, as previously described [8,9]. Cells were maintained at 33°C in a humidified atmosphere (95% air/5%CO_2) using growth medium [α-MEM containing 10% FBS and 1% PS]. Rat osteosarcoma cells ROS 17/2.8, human osteosarcoma SaOS-2 and COS-7 cells were maintained at 37°C in a humidified atmosphere (95% air/5%CO_2) using growth medium (Ham's F-12 for ROS 17/2.8 and DMEM for SaOS-2

and COS-7) containing 7% FBS and 1% PS. Medium was changed every 2–3 days and cells were subcultured weekly using trypsin/EDTA. For radioligand binding and crosslinking experiments, ROS 17/2.8 cells were treated for 48 h with 0.5 mM 8Br-cAMP to increase their CPTHR expression, as previously reported [15].

2.3 Radiolabeling of peptides

Radiolabeled hPTH(19–84), Bpa^{24}-hPTH(23–84), and Bpa^{26}-hPTH(24–84), were prepared by chloramine-T iodination, followed by high performance liquid chromatography purification using a 30–50% acetonitrile gradient in 0.1 % trifluoroacetic acid over 30 min as previously described [4].

2.4 Isolation of cell membranes

OC59 cells were plated at a density of 10^5 cells/ml in 10 cm culture dishes and cultured at 33° C for approximately 3 weeks prior to membrane isolation. The cells were washed several times with PBS to remove the media components and scraped with a disposable cell scraper (Fisher). A 50% (v/v) suspension of cells was prepared in PBS with protease inhibitor cocktail (Sigma) and passed 8 to 10 times through a ball-bearing homogenizer (HGM, Heidelberg) to completely disrupt the cells. The lysate was centrifuged at 1000 rpm for 10 minutes to pellet the nucleus and intact cells and subsequently centrifuged at 100,000 rpm to obtain the membrane pellet. Unless used immediately, the membrane pellets were snap-frozen in liquid nitrogen and stored at −80 °C. Protein concentration was determined with the BCA assay system (Pierce) using the manufacturer's protocol.

2.5 Radioligand-receptor binding assay

Binding assays on intact cells were performed as described previously [4,8]. In brief, cells were plated in 24-well plates at 10^5 cells/ml and cultured at 33°C for approximately 3 weeks, for OC59, or at 37°C for 5–7 days, for ROS17/2.8 and SaOS-2. Each well (final volume of 500 μl) contained binding buffer [100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 50 mM Tris-HCl (pH 7.8) plus 5% heat-inactivated horse serum], ^{125}I-labeled ligand (approximately 10^5 cpm/well), and varying concentrations of unlabeled peptide. After 4 h at 16°C, the binding mixture was removed, and the cells were rinsed three times with 500 μl/well of cold binding buffer and lysed with 500 μl of 1M NaOH. Aliquots (400 μl) of the lysate then were counted for γ-irradiation. Specific binding was determined after subtracting radioactivity bound in the presence of an excess of unlabeled peptide (1μM). In some experiments, cells were extensively washed with acidic buffer (50 mM glycine, 150 mM NaCl, pH 2.4) to remove ligand bound to the cell surface.

Alternatively, binding was detected on nonadherent cells using nonradioactive Bpa^{24}-hPTH (23–84). Briefly, cells were washed twice with ice cold PBS, incubated with 0.5 mM EDTA for 20 minutes at 4°C and then re-suspended in 500 μl binding buffer. Cells (10^6 in 500 μl binding buffer) then were incubated with Bpa^{24}-hPTH(23–84) (500nM) and 1 μl Phycoerythrin (PE) streptavidin (Guava Technologies) for 30 minutes at 4°C. Cells were then centrifuged at 500 rpm for two minutes at room temperature and the cell pellets washed twice with ice cold PBS and resuspended in 500 μl binding buffer. Cell-associated fluorescence was analyzed by Guava Express with a GUAVA PCA system (Guava Technologies). A fluorescent Intensity threshold of "10^1" (PM1 = PE intensity) was arbitrarily assigned based on the non-specific fluorescence present in cells not treated with PE.

For membrane binding, the membrane pellet(s) was homogenously resuspended in PBS containing 5μg/ml BSA by repeatedly passing the suspension through a 25G needle. Approximately 15–20μg protein equivalent of the membrane suspension was used for each experimental condition. In one experiment, specific and total binding was determined by

incubating the membrane with increasing amounts of ^{125}I-Bpa^{24}- hPTH(23–84) in the presence or absence of excess (1μM) unlabeled Bpa^{24}-hPTH(23–84). Alternatively, specific ligand displacement was determined by incubating the membranes with 10^5 cpm of ^{125}I-Bpa^{24}-hPTH (23–84) in the presence of increasing concentrations of unlabeled Bpa^{24}-hPTH(23–84), or high concentrations of different previously characterized PTH peptides. All incubations, washing and radioactive assays were carried out in 96 well Multiscreen units (cat# MADVN0B10; Millipore). Washing, tracer and peptide dilutions were carried out in PBS containing 5μg/ml BSA as mentioned above, using a Millipore Multiscreen vacuum manifold. Upon completion of the incubations, the Multiscreen filter discs were air-dried, plucked manually from the frame and counted for γ-irradiation.

2.6 Determination of $[Ca^{2+}]_i$

[Ca2+]i was monitored by ratio microfluorimetry using the fura-2 method[14]. In brief, cells were plated into Lab-Tek II chambered cover glasses (Nalge Nunc International, Naperville, IL) at a density of 10^4 cells per well and cultured as described above. For measurement of $[Ca^{2+}]$i, cells were transferred to room temperature and washed with a balanced salt solution (BSS; 127 mM NaCl, 3.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES, pH 7.4) before loading with 5 μM fura-2 acetoxymethyl ester (with 0.05% Pluronic F-127; Molecular Probes, Eugene, OR) for 45 min at room temperature, washing in balanced salt solution, and further incubation at room temperature for 30 min to allow discharge of uncleaved fura-2AM. In cells prepared in this manner, fura-2 fluorescence was diffuse without a punctate distribution pattern. Fluorescence was measured in single cells excited alternatively at 340 and 380 nm using a PTI Deltascan dual-wavelength fluorimeter (Photon Technologies Incorporated, Lawrenceville, NJ) directed through the stage of an inverted Nikon Diaphot 200 microscope (Melville, NY) with a cut-off filter at 400 nm. Emissions were monitored in real time with a Sensys charge-coupled device camera (Photometrics, Ltd., Tucson, AZ) and analyzed using the Poenie-Tsien ratio[14] with Imagemaster 2 software (Photon Technologies Incorporated, Lawrenceville, NJ). At the conclusion of each experiment, fura-2 fluorescence was calibrated to $[Ca^{2+}]$i by treating the cells with 5 μM ionomycin to achieve the maximum Ca^{2+}-bound dye ratio at 340/380 nm and fluorescence at 380 nm excitation (R_{max} and S_{b2}), followed by treatment with 5 mM EGTA to determine Ca^{2+}-free dye ratio at 340/380 and fluorescence at 380 nm excitation (R_{min} and S_{f2}). These parameters were used to estimate [Ca2+]i using the equation of Grynkiewicz *et al* [14]. Calcium transients monitored in the absence of extracellular calcium are reported as a ratio of fura-2 fluorescence at 340 nm excitation over that at 380 nm.

2.7 Photoaffinity crosslinking of the CPTHR

For photoaffinity labeling, cells were plated in 6-well dishes or in 10-cm dishes at a density of 10^5 cells/ml and cultured as described above. Cells were washed once with ice-cold binding buffer and incubated for 4 h at 16°C with ^{125}I-Bpa^{24}-hPTH(23–84) or ^{125}I-Bpa^{26}-hPTH(24–84) (10^6 cpm/well) protected from light. After this incubation with ligand, cells were rinsed three times with ice-cold buffer before adding 0.8 ml (for 6-well dishes) or 5 ml (for 10 cm dishes) of binding buffer and placing the dishes on ice under a UV light source for 15 min (Black Ray long-wave lamp; 366nm, 700 microvatts/cm^2; UV products, San Gabriel; source-to-cell distance of 5 cm). After UV crosslinking, cells were rinsed twice with ice-cold binding buffer to remove non covalently bound radioligand, solubilized with 1% Triton buffer (50mM Tris-HCl, pH 7.8, 1% Triton X-100, 50μg/ml bacitracin, 10mM PMSF, 140 mM NaCl) and centrifuged at 13000 rpm for 10 min. The supernatant was then mixed 1:1 with 2X SDS-PAGE sample buffer to give the final concentration of 4% SDS, 80 mM Tris-HCl (pH 6.8), 20% glycerol, 0.2 % bromphenol blue and 10mM β-mercaptoethanol, as previously described [11]. In some experiments the radioligand binding assay was carried out in binding buffer containing an excess of biotin (100 mM) to saturate avidin-like binding sites.

For analysis of ligand-receptor crosslinking in membranes, the Multiscreen filter discs from the previous section were moistened with PBS, exposed to long wavelength UV radiation for 15–20 minutes at 4°C and subsequently incubated with Laemmli sample buffer for 30 minutes at 70°C in a dry heating block before analysis by SDS-PAGE.

2.8 Analysis of the CPTH crosslinked complexes

The samples in SDS-PAGE sample buffer were incubated at room temperature for 1–2 h and then subjected to SDS-PAGE analysis (7.5% or 10% acrylamide) performed according to the method of Laemmli for intact cell binding or membrane binding and crosslinked samples. For visualization of the intact crosslinked products, the gels were dried and subjected to autoradiography at −80°C for a variable amount of time. Alternatively, the crosslinked products were run on a 10% SDS-PAGE, electroblotted to nitrocellulose membrane and subjected to western blot analysis using Streptavidin-HRP (Sigma) to detect the biotinylated peptide.

Both UV (photoaffinity) and chemical methods were used to verify crosslinking between CPTH peptides and the receptor proteins. Binding was performed with ^{125}I-Bpa^{24}-hPTH(23–84) on OC59 cells as mentioned above. One set of cells was crosslinked using UV for 15 minutes at 4°C. A combination of both chemical and photoaffinity crosslinking was done by incubating a second set of cells with 100 mM Sulphosuccinimidyl–4- (p-malimidophenyl) butyrate (Sulpho-SMPB; Pierce) in binding buffer and exposing the cells to UV for 30 minutes. Chemical crosslinking was performed with another set of cells using 100 mM sulpho-SMPB in binding buffer and incubated at 4 °C for 30 minutes with rotation. The cells were scraped and directly solubilized in 1X SDS-PAGE sample buffer, analyzed by 10% SDS-PAGE and exposed to X-ray film.

For isolation of the crosslinked ligand-receptor complexes, the peptide-bound and UV-crosslinked cells were lysed with 1% sodium deoxycholate in PBS for 1 hour at room temperature with gentle rotation. The volume of the lysate was increased with PBS to bring the final concentration of deoxycholate to 0.1%. The insoluble material in the lysate was removed by centrifugation and the clear lysate was incubated with goat anti-hPTH(39–84) antibody, immobilized on 4% beaded agarose (Immunotopics), pre-equilibrated with PBS, for 30 minutes at room temperature. The matrix was washed extensively with PBS, and elution of the bound complexes was carried out with 0.1M glycine, pH 2.5 with 0.1% SDS. The pH of the elute was restored to physiological range with unbuffered 2M Tris solution. As a parallel approach, a two-step purification of the CPTH-protein complex(es) was carried out. The elute from the antibody affinity column was diluted 10-fold with 50 mM phosphate-citrate buffer, pH 4.0 and incubated with CaptAvidin-Agarose matrix (Molecular Probes) for 30 minutes at room temperature with mild rotation. The matrix was washed extensively with the binding buffer and the bound proteins eluted with 50 mM sodium bicarbonate, pH 10.0. Elutes from both one-step and two-step purifications were concentrated by lyophilization or precipitation [23] before further analysis.

The antibody affinity-purified proteins were precipitated using the methanol/chloroform phase precipitation method [23], resuspended with a minimum volume of 6M guanidium hydrochloride solution and incubated at room temperature for 1 hour. The protein complexes were recovered by overnight dialysis against PBS with three changes of the buffer and subsequent lyophilization. Simultaneously, the antibody affinity-purified proteins were run on a 10% preparative SDS-PAGE and gel strips corresponding to 220kDa and 80kDa mol. wt. ranges were excised for electroelution using a Bio-Rad model 422 electroelution apparatus and SDS-PAGE running buffer as the mobile phase. The samples were electro-dialyzed in the same apparatus by changing the buffer to 50 mM ammonium bicarbonate buffer, pH 8.0. All samples were further analyzed by SDS-PAGE and electroblotting, using streptavidin-HRP as a probe

for the latter. OC59 whole cell proteins were treated with 6M GuHCl and processed with the crosslinked sample as the negative control.

In a separate experiment, OC59 total membrane (10μg protein/experimental condition) was incubated with increasing amounts of ^{125}I-Bpa^{24}-hPTH(23–84) (0 to 10μM) and UV-crosslinked. Another set of samples were incubated with 10^5 cpm of ^{125}I-Bpa^{24}-hPTH(23–84) and competed with increasing concentrations of non-radiolabled Bpa^{24}-hPTH(23–84) (0 to 10μM) and run on a 12% SDS-PAGE after UV crosslinking. The dried gel then was exposed to X-ray film.

2.9 Statistical Analysis

Results were expressed as the mean ±SD or SE. Each experiment was repeated three to five times. Significance of differences between treatment and control groups was assessed by one-way ANOVA using Bonferroni correction and Prism 3 software (GraphPad, San Diego, CA).

3. RESULTS

3.1 CPTHR binding of Bpa^{24}-hPTH(23–84) and Bpa^{26}-hPTH(24–84)

Initial analysis of the two biotinylated Bpa analogs, Bpa^{24}-hPTH(23–84) and Bpa^{26}-hPTH(24–84) was performed in competition-binding studies with the OC59 osteocytic cell line and rat osteosarcoma cells ROS17/2.8, both of which express abundant CPTHRs [8,15]. The two Bpa-containing PTH peptides were fully functional and exhibited binding affinities indistinguishable from that of hPTH(19–84), indicating that neither the Bpa substitution in position 24 or 26, nor the biotin at the carboxyl-terminus, interferes with high affinity binding (Fig. 1). As shown in Figure 1 and Table I, the apparent IC_{50}s of hPTH(19–84), Bpa24-hPTH (23–84), Bpa^{26}-hPTH(24–84), hPTH(23–84) and hPTH(24–84) were all in the range of 20–80 nM, as previously reported for the intact hormone [7,15]. All peptides exhibited a total binding comprised between 6000 and 9000 cpm (6410 ± 2200 cpm) and a non specific binding between 900 and 1900 cpm (1378 ± 442 cpm). Moreover, when the Bpa-containing peptides were used as radiotracers (Fig. 1 B, C, E and F), they were indistinguishable in terms of binding from ^{125}I-hPTH(19–84) (Fig. 1 A and D), indicating that all of the peptides tested are likely binding to the same receptor site(s).

To further analyze the binding properties of the biotinylated PTH fragments, we used fluorescence cytometry (Guava Express, Guava Technologies) to detect the expression of CPTHRs on OC59 cells. Cells were incubated with 0.5μM Bpa^{24}-hPTH(23–84) and PE-streptavidin for 30 min at 4°C. Under these conditions, about 90% of the cells (average of 1817 high-fluorescence events out of a total of 2000 counted) were “positive” for CPTHR expression, compared to approximately 4% (average of 83 high-fluorescence events out of 2000 counted) in cells not exposed to biotinylated ligand or in cells treated with PE-streptavidin alone (Fig. 2B). To assess the specificity of this binding, we performed the same experiment in COS-7 cells that do not express CPTHRs, as previously reported [8,15]. As expected, after incubation with 0.5 μM Bpa^{24}-hPTH(23–84) and PE-streptavidin, only 6% of the COS-7 cells exhibited suprathreshold fluorescence, consistent with the other negative controls above (Fig. 2E).

As shown in Fig. 2 D & F, binding of Bpa^{24}-hPTH(23–84) was competitive with that of added hPTH(1–84). Intact hormone displaced Bpa^{24}-hPTH(23–84) in a dose- dependent manner with an apparent IC_{50} between 5 and 50nM (Fig. 2G), which is very similar to that estimated by radioligand binding (Table I).

3.2 Binding assays with the membrane

To ascertain if specific CPTHR binding is present in OC59 membrane preparations, membranes were isolated and radioligand binding assays were conducted using ^{125}I-Bpa^{24}-hPTH(23–84) as tracer and various CPTH analogs as competitors. Saturable, specific binding of the tracer was sought by adding increasing amounts of radiolabeled tracer in the presence vs. absence of 1μM non-radioactive Bpa^{24}-hPTH(23–84) (Fig. 3A). Competition analysis (Fig, 3B) showed that the apparent IC_{50} of binding to membranes was >100nM when the tracer was competed with excess (0 to 10μM) of non-radioactive Bpa^{24}-hPTH(23–84), several-fold higher than that observed using intact cells (Fig. 1, Table I). Results of competition assays with CPTH peptides were almost similar to those of the whole cell assays, except that the non-specific binding was found to be higher than the intact cell assays. Human PTH(1–34) (1μM), as expected, minimally displaced the ^{125}I-Bpa^{24}-hPTH(23–84) tracer, whereas the intact hormone (1–84; 1μM), as well as Bpa^{24}-hPTH(23–84) (1μM), did significantly displace the tracer. Human PTH(53–84) (10μM), with or without biotin at the C-terminus, showed similar displacement of the tracer in OC59 membranes, thus demonstrating that the presence of a biotin at the C-terminus does not affect the hormone-receptor interaction (Fig. 3C).

3.3 Determination of $[Ca^{2+}]_I$

We recently reported that CPTHR induces a rapid increase in intercellular calcium, mostly due to influx from the extracellular pool [22]. To investigate if the modified CPTH peptides still retain functional activity, we measured calcium influx in OC59 cells in response to CPTH treatment. As expected (Figure 4B), 250 nM Bpa^{24}-hPTH(23–84) induced a rapid transient elevation of $[Ca^{2+}]_I$, that reached a maximum within 20 seconds after peptide addition and returned to the baseline 10–15 seconds thereafter, which was comparable with the effect previously described for hPTH (53–84) [22]. Similar calcium responses were observed with 10 and 50 nM Bpa^{24}-hPTH(23–84) (data not shown).

Addition of vehicle alone (buffered solution) had no effect, indicating that the increase in $[Ca^{2+}]_I$ was specific for the peptide and not due to a mechanical stimulation of the cells (Figure 4A).

3.4 Analysis of the crosslinked complex(es)

In preliminary experiments, intact OC59 cells or crude membrane preparations were incubated with ^{125}I-Bpa^{24}-hPTH(23–84) for different times and incubation temperatures to investigate the kinetics of hormone-receptor interaction. Negligible differences in the amounts of specific binding were observed after 2 and 4 hours at room temperature, 4 hours at 16°C or 16 hours at 4°C, suggesting that the hormone interaction with the receptor was quite rapid. We also sought evidence of internalization of radioligand by intact cells, as assessed by recovery of previously bound radioligand with an acidic buffer wash. Cells were incubated for 1 and 2hr at 4°C, 16°C or room temperature, washed with binding buffer and then washed with glycine buffer (pH 2.5) to recover bound radioligand still present on the cell surface. These experiments suggested that approximately 50% of the radioligand was internalized at room temperature vs. only 20% when cells were incubated at 4° or 16°C (data not shown).

In other preliminary studies, crosslinking of ^{125}I-Bpa^{24}-hPTH(23–84) to OC59 cells was analyzed by SDS-PAGE and autoradiography following UV photoactivation, chemical crosslinking with sulpho-SMPB (16), or both. In each case, similar patterns of radiolabeled proteins were observed (see below). As the highest efficiency of crosslinking was observed with UV photoaffinity labeling, this method was employed in all subsequent experiments. Photoaffinity crosslinking of ^{125}I-Bpa^{24}-hPTH(23–84) or ^{125}I-Bpa^{26}-hPTH(24–84) to OC59 cells, followed by direct SDS-PAGE of the detergent-solubilized washed-cell extracts, showed a predominant high-molecular weight (> 200kDa) species and evidence of smaller complexes

at 80kDa and 30–40kDa mol. wt. ranges (Figure 5A). Similar results were observed using the osteoblastic cell lines, ROS 17/2.8 and SaOS-2, which are known to express various levels of CPTHR [8, 15]. In ROS17/2.8 cells, the intensities of the radioactive bands were similar to those in OC59, while in SaOs2 cells the proteins were barely detectable (Fig. 5A). In all three cell lines, however, excess nonradioactive hPTH(1–84), but not hPTH(1–34), could readily displace the iodinated hormone, confirming the specificity of these bands. Identical results were obtained with ^{125}I-Bpa^{24}-hPTH(23–84) and ^{125}I-Bpa^{26}-hPTH(24–84) suggesting that the two analogs have similar properties. Further studies were conducted with ^{125}I-Bpa^{24}-hPTH (23–84) only (Fig. 5A).

Crosslinking of ^{125}I-Bpa^{24}-hPTH(23–84) to OC59 cell membranes, followed by SDS-PAGE and detection by either autoradiography or Western Blotting, probing with Streptavidin-HRP, showed similar results (Figure 5B), with 220kDa, 80kDa and 30kDa proteins identified as putative CPTH-protein complexes. The specificity of the binding of ^{125}I- Bpa^{24}-hPTH(23–84) for the 80kDa protein was further probed by demonstration of competitive displacement with nonradiolabeled Bpa^{24}-hPTH(23–84) (Figure 5C).

3.5 Isolation of the CPTH-protein complex(es) from the cell lysate

To investigate if Bpa^{24}-hPTH(23–84) peptide might be used to purify the hormone-receptor complex, two different affinity matrices (anti-hPTH 39–84 IgG-Agarose and/or CaptAvidin-Agarose) were employed individually or in combination. For solubilizing the receptor(s) from plasma membranes, three different detergents/chaotrophs (1% deoxycholate; 1% CHAPS or 1% Triton X-100 in PBS) were tested in preliminary experiments for solubilization of the cross-linked material and compatibility with the two affinity matrices. Among the three, 1% deoxycholate (1% DOC) was found to be the most effective in these respects and was employed subsequently.

For the experiment shown in Figure 6, OC59 cells were bound to nonradioactive Bpa^{24}-hPTH (23–84) and UV cross-linked. The cells were then solubilized with 1% DOC in PBS, diluted with PBS and allowed to bind to the antibody matrix. After elution from the matrix with acidic buffer, the affinity-purified fraction was analyzed by SDS-PAGE and electroblotted for streptavidin-HRP detection. As shown in Figure 6B (lane 1), the antibody-affinity purified sample showed the same 220kDa, 80kDa and 30kDa bands previously detected after direct analysis of cell lysates (Figure 5B). Note that the two unmarked bands (at approximately 130kDa and 66kDa), are unrelated to the CPTHR, as they appear when the experiment is performed in the absence of the biotinylated CPTH ligand (Fig. 6B lane *3*). Upon direct purification over the CaptAvidin-Agarose matrix, however, the solubilized crosslinked ligand/receptor complex migrated mainly at 80kDa, with a faint band at 30kDa mol. wt. range and no detectable species at 220kDa (data not shown). A similar result to that was obtained when the antibody-affinity-purified fraction was subsequently processed over the CaptAvidin matrix (Fig 6B lane *2*). Despite the fact that the antibody-affinity-purified fraction contained relatively little protein (see Figure 6A, lane 4), these results suggested that the 220kDa species might reflect aggregation of the CPTH/CPTHR complex in the conditions employed for binding, crosslinking or subsequent analysis.

3.6 Analysis of the 220kDa mol.wt. complex

To further investigate the possibility that the 220kDa protein band observed in the antibody-affinity eluate might consist of aggregates of CPTH/CPTHR proteins, either with one another or with other protein(s), antibody affinity-purified material was run on a preparative SDS gel and the strips corresponding to the 220kDa and 80kDa protein bands were separately excised. The proteins in those bands then were electroeluted and concentrated by methanol-chloroform precipitation. The salt-free and air-dried protein pellets were dissolved in 6M Guanidium

hydrochloride (GuHCl) to separate the component proteins of the complex (if any) and then subjected to SDS-PAGE and electro blotting using Streptavidin-HRP detection. As seen in Fig. 7, while the GuHCl-treated 80kDa complex again migrated in the expected molecular weight range (right lane), the 220kDa protein band, after GuHCl treatment, now migrated at 80kDa apparent mol. wt. range (middle lane). Again, the bands seen at 130kDa and 66kDa ranges are nonspecific and unrelated to the CPTHR, as they were detected in GuHCl-treated OC59-cell proteins in the absence of biotinylated CPTH ligand (Figure 6A, left lane). This result suggested that the 220kDa complex, earlier considered to be one of the native CPTH-CPTHR complex, was indeed a transient aggregate of various proteins, which includes the 80kDa putative crosslinked ligand/receptor species.

4. DISCUSSION

In this study we report the synthesis and characterization of two novel PTH peptide analogs that specifically bind and crosslink to the CPTHR. Our previous studies, using sequentially N-terminally truncated CPTH peptides, showed the existence of discrete ligand domains containing residues critical for CPTHR binding within the intact hormone[7]. Following this initial observation, we designed two peptides containing a modified photoreactive alanine derivative that, upon activation by UV light, can be covalently crosslinked to lysines/methionines present on the CPTHR [3,13,18]. In addition, we added a biotin at the carboxyl-terminal end of the peptide to enable avidin-based detection and isolation. Here we report that the two modified PTH analogs, Bpa^{24}-hPTH(23–84) and Bpa^{26}-hPTH(24–84), can specifically bind and crosslink to the CPTHR and that the resulting ligand/receptor complex can be partially purified using anti-PTH antibody or CaptAvidin as affinity reagents. Initial analysis of the purified proteins revealed the presence of three distinct complexes of approximate mol. wt. of 220kDa, 80kDa and 30kDa. Subsequent resolution of these proteins by gel purification after exposure to chaotrophs has demonstrated that the 220kDa protein most likely is a non-covalent aggregate that can be resolved into the 80kDa ligand-containing moiety.

Our studies have shown that while it is possible to saturate all the putative CPTH binding sites with increasing concentrations of the CPTH peptide(s), it may only be possible to effect a cross-linking efficiency in the vicinity of 1 % of the total receptors present, as assessed by measuring the radioactivity retained by the putative receptor bands in an SDS-PAGE gel with respect to the total radioactivity bound (applied on the preparative SDS-PAGE) (data not shown). To further evaluate the properties of receptor-ligand interaction, we analyzed membrane binding.

Binding and competition assays with isolated cell membranes revealed a similar profile as the intact cell assays, except that the IC_{50} values in the case of the membranes were found to be somewhat elevated. This could be attributed to some alteration in protein structure of the receptor molecules during the shearing stress or other procedures of membrane preparation. Alternatively, additional cytoplasmic proteins might be required for high affinity binding. The fact that the radioactive labeled peptide could be competitively dislodged from the membrane with excess non-radioactive peptide (both full length PTH and the non-radioactive version of the tracer) argued for the specificity of the experiment and suggested use of membranes as a source of receptor for a quantitative scale-up of the experiment. The ligand selectivity of the membrane CPTHRs appeared to be similar to that seen in intact cells, in that hPTH(1–34) showed minimal interaction whereas hPTH(53–84) did effectively compete with the radiolabeled Bpa^{24}-hPTH(23–84) ligand. Studies with hPTH(53–84) with or without the C-terminal biotin indicated no major effect of the biotin moiety on receptor binding (Fig. 3C).

Initial biochemical studies with the ‘Bpa’-mediated cross-linked cells/membranes revealed three putative complexes (220kDa, 80kDa and 30kDa apparent mol. wt.) for the CPTHR(s) in OC59 cells. This data was consistent for both radioactive and streptavidin-HRP electroblot

detection. However, upon two-step purification, or single-step purification directly with CaptAvidin-Agarose, the 220kDa band was seen to disappear from all analytical results. Although surprising, this phenomenon was later shown to be due to the formation of a transient complex of the 80kDa protein, possibly with certain other cellular/membrane proteins, which could be dissociated in the presence of a strong chaotroph like guanidium hydrochloride. The 220kDa band was seen to dissociate into components, which included the 80kDa, but not the 30kDa complex. The results obtained were also consistent with the results previously reported in ROS 17/2.8 using SMPB-crosslinking(16).

Thus, incorporation of Bpa and biotin into the CPTH and use of OC59 cells (PTH1R −/−) may be powerful tools with which to purify the CPTHR(s) in osteocytes, with a potential for easy cross-linking and identification of similar receptors from different other cell types. As reported for the PACAP receptor[21], it could be feasible to identify the CPTHR after isolation and mass-spectroscopic identification. There are, however, a few optimizations required in the study presented here before the final identification of the receptor(s), all in the field of detection. The use of streptavidin-HRP for detection, while on one hand, considerably decreases the analysis time compared to the lengthy radioactive detection, also exhibits false positive bands, sometimes more prominent than the specific bands of the CPTH-complex(es). The case in point is the appearance of two bands of approximately 130kDa and 66kDa in the antibody-affinity elutes (Fig. 5B), which are also present in the CPTH-untreated cell/membranes, treated with 6M Guanidium hydrochloride (Fig. 6A).

In summary, we have reported the synthesis and characterization of two large modified CPTH peptides containing a Bpa substitution and a biotin at the C-terminus. Initial analysis of these two peptides revealed that they are functionally identical to the native hormone with respect to their ability to bind to CPTHRs on both clonal osteocytes and osteoblastic osteosarcoma cells. Two-step purification demonstrated that these peptides can covalently bind to the CPTHR upon photoaffinity crosslinking and thus could be used as to purify the hormone-receptor complex and potentially identify it by mass-spectrometric analysis. Although the molecular identity of the CPTHR is still unknown, we now have powerful new tools with which to approach the identification of this protein.

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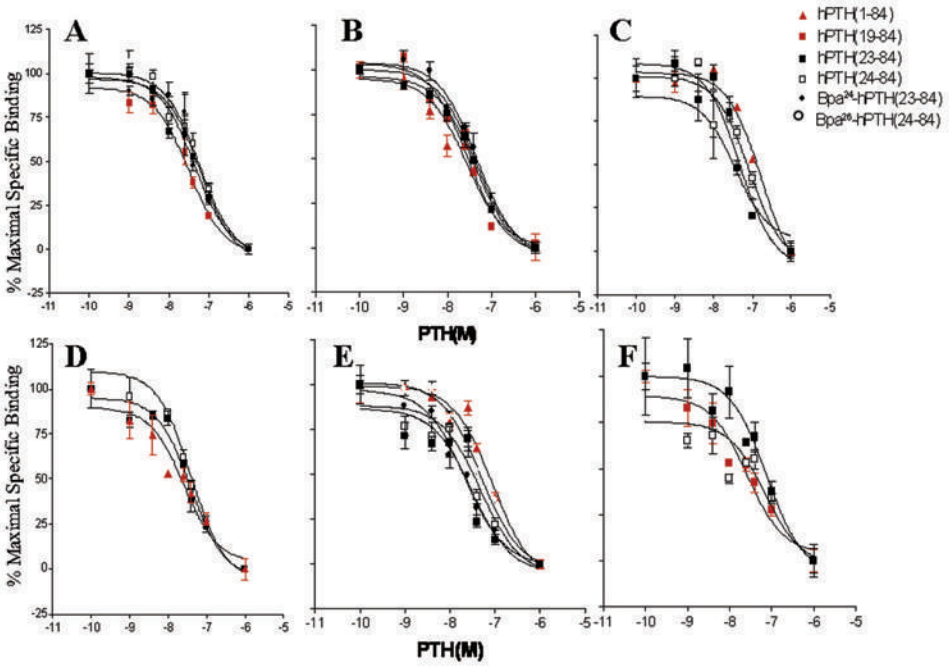


Figure 1. Binding of biotinylated PTH analogs to CPTHRs on OC59 and ROS 17/2.8 cells

OC59 cells (panels A–C) and ROS17/2.8 cells (panels D–F) were seeded at 10^5cell/ml in 24-well plates, cultured and used in radioligand binding assays as described in Experimental Procedures. Displacement of different radioligands - ^{125}I-hPTH(19–84) (panels A & D), ^{125}I-Bpa^{24}-hPTH(23–84) (panels B & E) or ^{125}I-Bpa^{26}-hPTH(24–84) (panels C & F) - by the indicated nonradioactive PTH peptides is seen as the reduction of maximal specific binding, after correcting for nonspecific binding observed in the presence of excess (10^{-6} M) hPTH(1–84). Results are expressed as the mean ± SD (n=3) of the percentage of maximal specific binding observed in the absence of competing ligand.

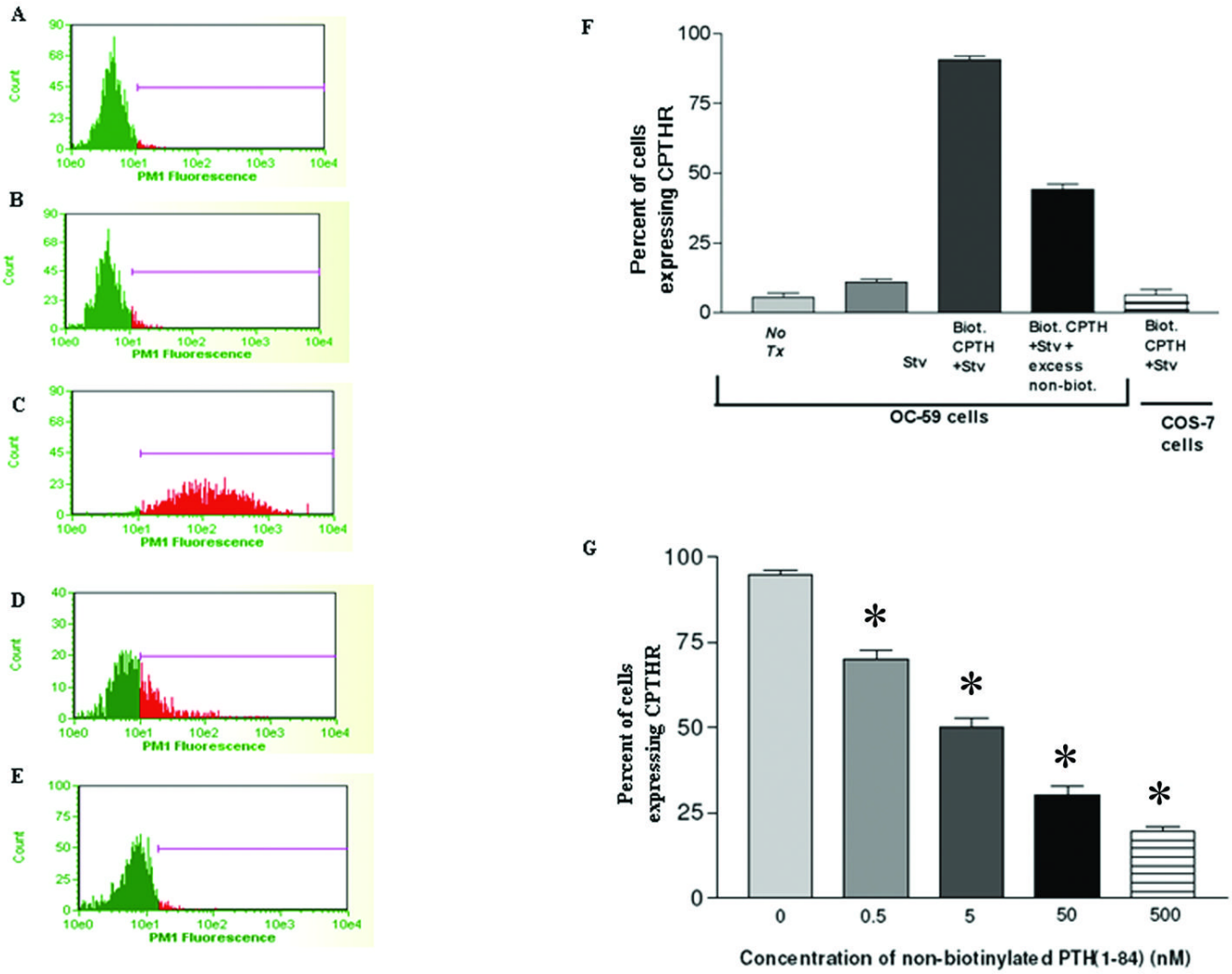
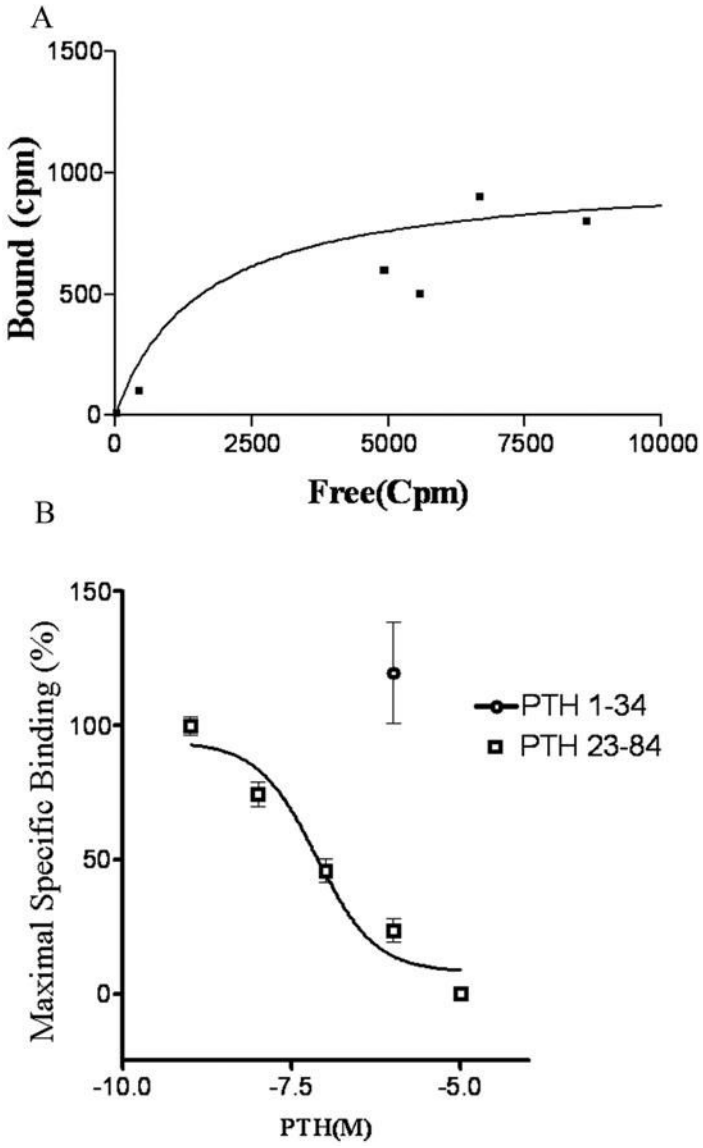


Figure 2. Assessment of CPTHR expression by cell-associated fluorescence

Cells were incubated with or without PTH peptide(s) and Phycoerythrin (PE)-streptavidin for 30 minutes at 4°C, centrifuged at 500 rpm for two minutes at room temperature, washed twice with ice cold PBS, and then re-suspended in 500 μl binding buffer for analysis of cell-associated fluorescence, as described in Methods. Histograms in panels A-E indicate the fraction of cells with associated fluorescence above a threshold (horizontal bar) exceeded by <5% of control cells (i.e. panels A & B). Panels **A–D**: OC59 cells were incubated with **(A)** Bpa^{24}-hPTH(23–84) (0.5μM) alone, **(B)** PE-Streptavidin alone, **C**- Bpa^{24}-hPTH(23–84) (0.5μM) plus PE-Streptavidin or **(D)**- Bpa^{24}-hPTH(23–84)(0.5μM), streptavidin and 1μM PTH(1–84). In panel (E), COS-7 cells were incubated with Bpa^{24}-hPTH(23–84) (0.5μM) and PE-Streptavidin. The results of 3 independent experiments corresponding to panels A–E are summarized in panel **F**, which depicts the mean ± SE of the percentage of 20000 cells scored that exceeded the fluorescence threshold. Panel **G** shows the percentage of 2000 scored OC59 cells expressing high fluorescence (mean ± SE) after Bpa^{24}-hPTH(23–84) was displaced by hPTH(1–84) added at the indicated concentrations. (* = P<0.001)



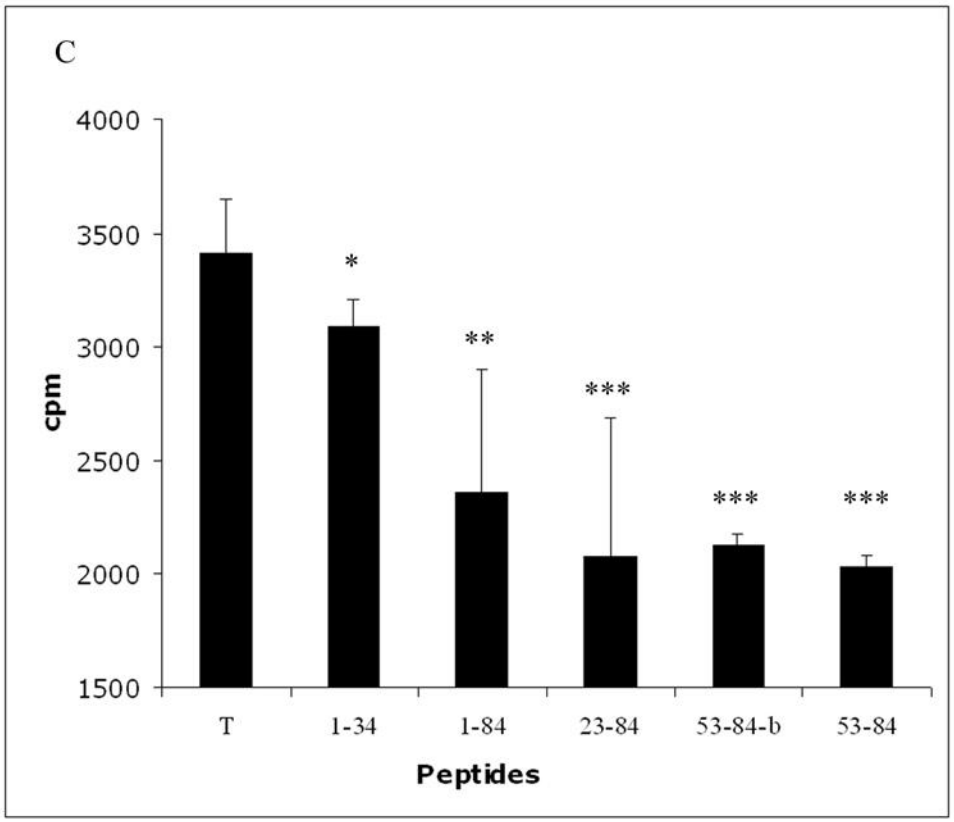


Figure 3. Binding to CPTHRs on membranes from OC59 cells

Membranes were isolated from OC59 cells and binding assays were conducted as described in Methods. (A) Plot of specifically bound vs. free radioactivity after addition of increasing amounts of ^{125}I-Bpa^{24}-hPTH(23–84) ± excess nonradioactive Bpa^{24}-hPTH(23–84). (B) Competitive displacement of ^{125}I-Bpa^{24}-hPTH(23–84) from OC59 membranes by increasing concentrations (0 to 10μM) of non-radioactive Bpa^{24}-hPTH(23–84), or 1μM hPTH(1–34). Results are expressed as the mean ± SE (n=3) percentage of the maximal specific binding observed in the absence of competing ligand.

(C) Competitive displacement of I^{125}-Bpa^{24}-hPTH(23–84) from OC59 membranes by 1μM each of hPTH(1–34), hPTH(1–84), hPTH(23–84) (non-radioactive Bpa^{24}-hPTH(23–84)), or 10μM of C-biotinylated hPTH(53–84) or unmodified hPTH(53–84). * = P< 0.05; ** = P< 0.01; *** = P< 0.001.

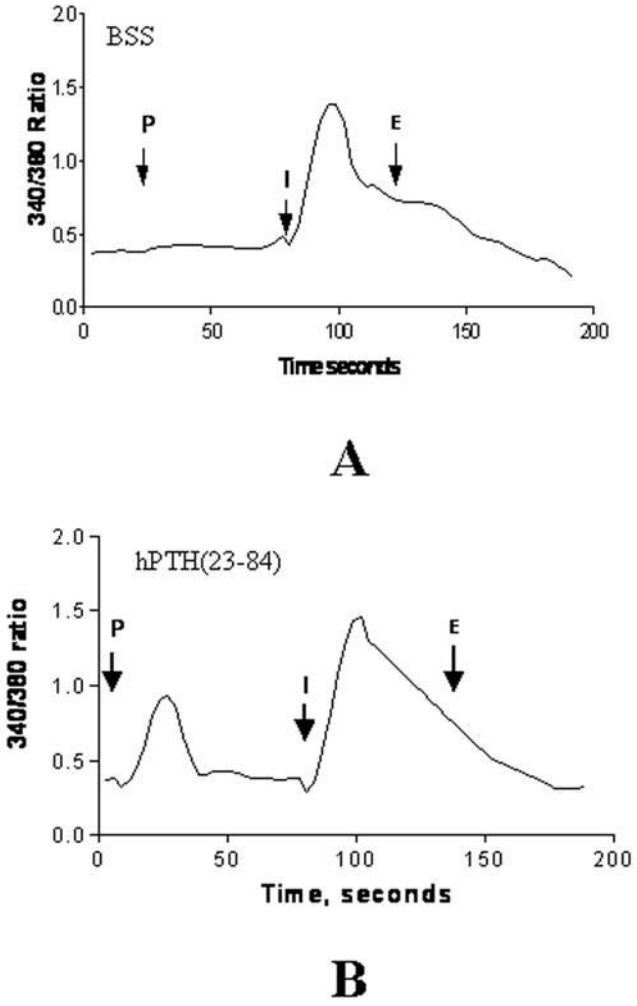


Figure 4. Regulation of [Ca^{2+}]i by hPTH(23–84) in OC59 cells

Ca_i++ was assessed by single-cell ratiometric microfluorimetry as described in Materials and Methods. Human PTH(23–84) (250 nM) (or vehicle alone) was added at the time indicated by the arrow (“P”). Subsequent calibration was performed by addition of ionomycin (“I”, 5 mM) followed by EGTA(“E”, 5 mM). (A) OC59 cells treated with vehicle alone; (B) OC59 cells treated with hPTH(23–84).

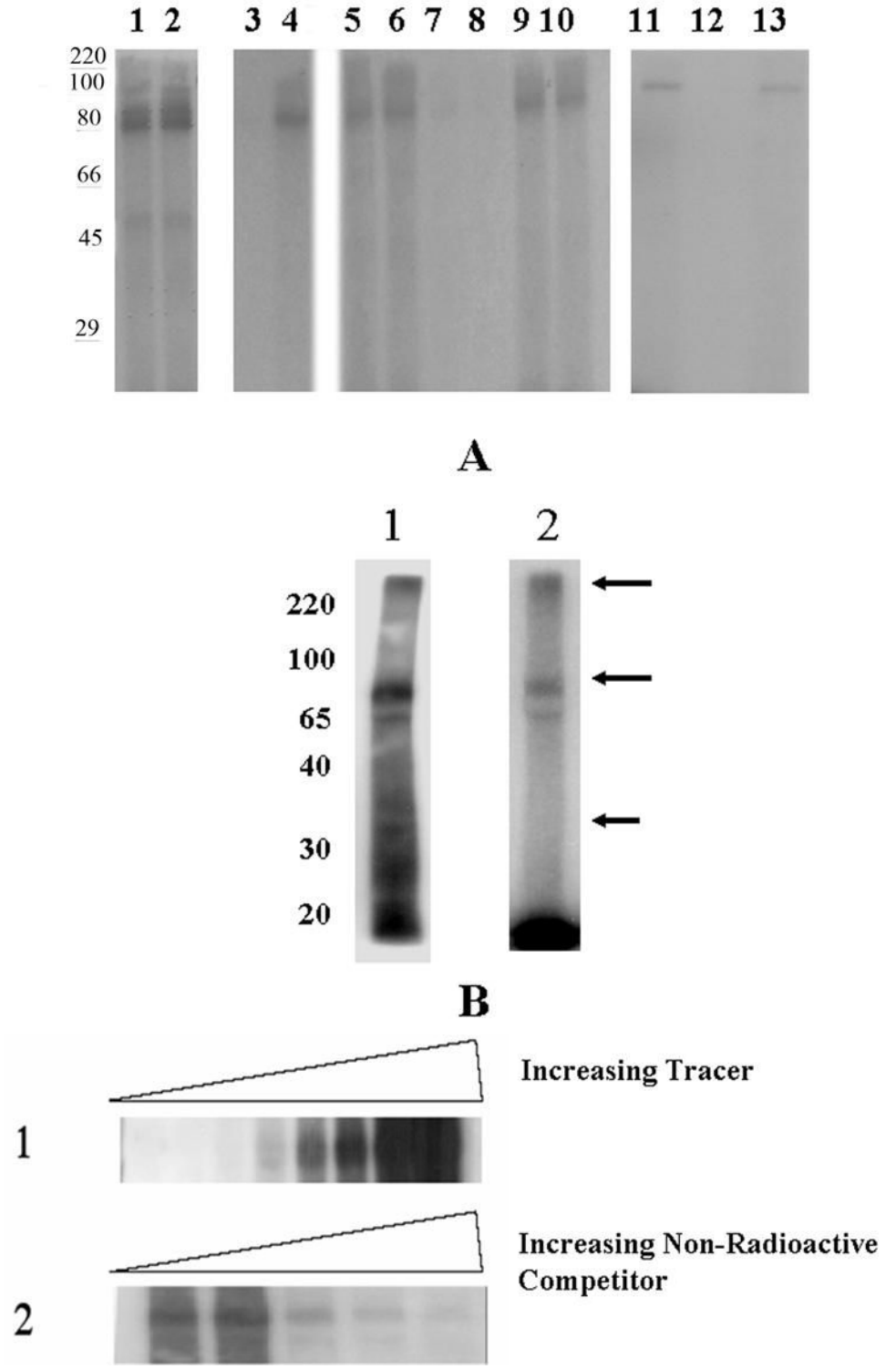


Figure 5. Analysis of CPTH analog crosslinking in bone-derived cells and membranes

(A) Cells were plated at 10^5 cells/cm^2 in 24 well-plates and cultured for various intervals at 33°C (OC59) or 37°C (ROS 17/2.8 and SaOS-2 cells) before whole-cell binding was carried out as described in Methods. Cells were then exposed to UV light for cross-linking, solubilized

and the proteins resolved by 12% SDS-PAGE. *Lanes* 1 and 2: OC59 cells cross-linked to ^{125}I-Bpa^{24}-hPTH(23–84). *Lanes* 3 and 4: ROS17/2.8 cells crosslinked to ^{125}I-Bpa^{26}-hPTH (24–84), in the presence (*lane* 3) or absence (*lane* 4) of 1μM hPTH(1–84). *Lanes 5–10*, OC59 cells crosslinked to ^{125}I-Bpa^{26}-hPTH(24–84), in the absence (*lanes 5,6*) or presence (*lanes 7,8*) of 1μM hPTH1–84) or hPTH(1–34) (*lanes 9 and 10*). *Lane 11–13*: SaOs2 cells, bound and crosslinked to ^{125}I-Bpa^{26}-hPTH(24–84), as described above, in the absence of competitor (lane 11) or in the presence of 1μM of hPTH (1–84) (lane 12) or hPTH(1–34) (lane 13).
(B) OC59 membranes were incubated with ^{125}I- Bpa^{24}-hPTH(23–84) and crosslinking was performed by incident UV radiation before analysis by 10% SDS-PAGE. Putative CPTHR(s) were detected either by transferring the proteins to Hybond-C extra membrane and subsequent western blot analysis with Streptavidin-HRP polymer (lane 1) or by direct exposure of the gel to X-ray film for detection of radioactivity (lane 2). Arrows correspond to 220 kD, 80 kD and 30 kD, respectively.
(C) *Panel 1*: OC59 membranes were incubated with increasing concentrations (0 to 10^5 cpm/10μg protein) of ^{125}I-Bpa^{24}-hPTH(23–84) and UV-crosslinked. The extracted proteins were separated on a 12% SDS-PAGE, ad the gel was then dried and exposed to X-ray film. *Panel 2*: OC59 membranes were incubated with a fixed amount of tracer (10^5 cpm/10μg protein) in the presence of increasing concentrations (0, 0.01, 0.1, 1, 10 μM) of non-radiolabled Bpa^{24}-hPTH(23–84) and binding analyzed as in Panel 1. Panels depict the 80kDa mol. wt. region of the gel.

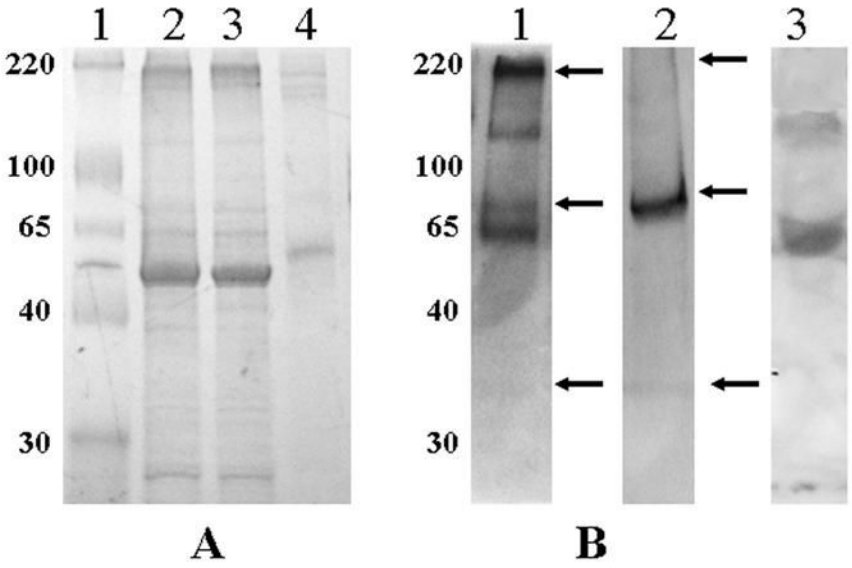


Figure 6. Affinity purification of CPTH/CPTHR complex(es)

Nonradioactive Bpa^{24}-hPTH(23–84) was incubated with OC59 cells and UV-crosslinked, cellular proteins were extracted with 1% deoxycholate/PBS and the clarified lysate was applied to a column of anti-PTH(39–84) agarose, as described in Methods. After washing, the CPTH peptide-associated protein was eluted with acidic glycine/SDS buffer. (A) Coomassie Blue-stained 10% SDS-PAGE gel. Lane 1: MW markers; Lane 2: total cell lysate before affinity purification; Lane 3: flow-through from antibody column; Lane 4: acid eluate of antibody column. (B) Streptavidin-HRP detection of the CPTH-crosslinked proteins on western blot. Lane 1: acid eluate of antibody column (corresponds to Lane 4 of panel A); Lane 2: the product of further purification of the antibody column eluate over Captavidin agarose (see Methods); Lane 3 : OC59 cell lysate from cells incubated without CPTH peptide, purified through anti-PTH antibody column, showing approximately 130 kDa and 66 kDa non-specific bands.

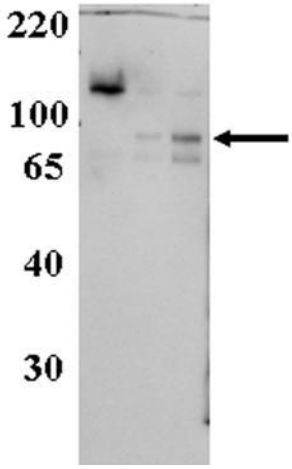


Figure 7. Disaggregation of antibody-purified 220 kD CPTH-crosslinked complex

The 220kDa and 80kDa bands obtained following preparative SDS-PAGE of antibody affinity-purified CPTH-crosslinked cell lysate (as in Figure 6B, Lane 1) were electroeluted from a 10% SDS-PAGE separately and treated with 6M guanidium hydrochloride. Subsequently, they were run on a 10% SDS-PAGE and analyzed on a western blot using Streptavidin-HRP as a probe. *Lane 1:* GuHCl-treated OC59 total proteins (no CPTH), *Lane 2:* Crosslinked 220kDa band after electroelution and GuHCl treatment and *Lane 3:* Crosslinked 80kDa band after electroelution and GuHCl treatment.

Table I

CPTHR binding in OC59 and ROS 17/2.8 cells[a]

IC_{50}(nM) OC59 cells	^{125}I-hPTH(19–84)	^{125}I-Bpa^{24}-hPTH (23–84)	^{125}I-Bpa^{26}-hPTH (24–84)
hPTH(1–84)	30.9 ± 15.1 *	63.1 ± 23.6 (n=7)	60.3 ± 43.3 (n=3)
hPTH(19–84)	37.8 ± 17.5 (n=5)	37.0 ± 28.2 (n=2)	nd
hPTH(23–84)	65.3 ± 26.2 (n=6)	42.2 ± 9.6 (n=4)	44.4 ± 15.7 (n=5)
Bpa^{24}-hPTH(23–84)	43.1 ± 28.9 (n=5)	61.3 ± 43.3 (n=3)	nd
hPTH(24–84)	55.5 ± 3.7 (n=3)	Nd	45.5 ± 37.7 (n=4)
Bpa^{26}-hPTH(24–84)	54.8 ± 18.8 (n=5)	66.25±30.75 (n=2)	56.6 ± 13.1 (n=3)
ROS17/2.8 cells	**^{125}I-hPTH(19–84)**	**^{125}I-Bpa^{24}-hPTH**	**^{125}I-Bpa^{26}-hPTH(24–84)**
hPTH(1–84)	21.7 ± 1.1 (n=3)	86.5 ± 4.9 (n=2)	nd
HPTH(19–84)	Nd	Nd	18.45±10.6(n=2)
hPTH(23–84)	35.0 ± 5.7 (n=2)	25.7 ± 16.1 (n=4)	54.5 ± 54.4 (n=2)
Bpa^{24}-hPTH(23–84)	Nd	24.3 ± 2.5 (n=3)	nd
hPTH(24–84)	21.7± 10.5 (n=5)	50.5 ± 31.0 (n=4)	56.40 ± 68.6 (n=2)
Bpa^{26}-hPTH(24–84)	Nd	60.1 ± 14.7 (n=3)	nd

[a] OC59 and ROS 17/2.8 were cultured as described in Experimental Procedures. Competitive displacement assays were performed using iodinated hPTH (19–84), Bpa^{24}-hPTH(23–84) and Bpa^{26}-hPTH(24–84) respectively and various hPTH peptides, as indicated in the table. Results are mean ± SD of several independent experiments (indicated in the tables). ND= not determined;

* = Previously published (14).