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Immunoprecipitation of the parathyroid hormone receptor

(immunoaffinity/antiligand antisera)

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ABSTRACT **An ^{125}I-labeled synthetic analog of bovine parathyroid hormone, [8-norleucine,18-norleucine,34-tyrosine]PTH-(1–34) amide ([Nle]PTH-(1–34)-NH_2), purified by high-pressure liquid chromatography (HPLC), was employed to label the parathyroid hormone (PTH) receptor in cell lines derived from PTH target tissues: the ROS 17/2.8 rat osteosarcoma of bone and the CV1 and COS monkey kidney lines. After incubation of the radioligand with intact cultured cells, the hormone was covalently attached to receptors by using either a photoaffinity technique or chemical (affinity) cross-linking. In each case, covalent labeling was specific, as evidenced by a reduction of labeling when excess competing nonradioactive ligand was present. After covalent attachment of radioligand, membranes were prepared from the cells and solubilized in the nonionic detergent Nonidet P-40 or octyl glucoside. The soluble membrane fraction present in the supernatant of a 100,000 × *g* centrifugation was incubated with IgG prepared from anti-PTH antiserum generated to the amino-terminal region, residues 1–34, of PTH. The IgG–PTH–receptor complex was precipitated with staphylococcal protein A-Sepharose. Analysis of the immunoprecipitate on NaDodSO$_4$/polyacrylamide gel electrophoresis followed by autoradiography revealed the presence of a doublet of apparent molecular mass 69–70 kDa. Specifically labeled bands of approximate molecular mass 95 and 28 kDa were also observed. The anti-PTH IgG was affinity purified by passage over a PTH-Sepharose column and used to make an immunoaffinity column. The 70- and 28-kDa bands were also observed after labeled solubilized membrane preparations were allowed to bind to this column and then were eluted by using a [Nle]PTH-(1–34)-NH_2-containing buffer or acetic acid. These studies suggest that the use of an anti-PTH antiserum that binds receptor-bound hormone is likely to be a useful step in the further physicochemical characterization and purification of the PTH receptor.**

Parathyroid hormone (PTH) binding to receptors on the plasma membrane of target tissue is thought to be the first step in expression of the biological activity of this calcium regulatory hormone (1). A receptor, or a subunit of the receptor, that specifically binds PTH has been identified as a 60- to 70-kDa (70 kDa is the size of receptor–hormone complex) membrane component in canine renal membranes by using photoaffinity techniques based on a biologically active photolabile radiolabeled PTH analog (2, 3). Subsequent studies demonstrated that a similar, if not identical, component was the principal plasma membrane constituent binding PTH in both human tumor-derived bone cells and skin fibroblasts (4).

However, further progress in characterization and initiation of purification efforts has been limited by the lack of availability of a highly resolving and high-capacity technique for separating the covalently linked hormone–receptor complex from other components of cellular membranes. In the studies described in this manuscript, we have developed immunoaffinity techniques for isolating PTH–receptor complexes.

MATERIALS AND METHODS

Materials. Bovine [(8-norleucine,18-norleucine,34-tyrosine]PTH-(1–34) amide ([Nle]PTH-(1–34)-NH_2) was purchased from Bachem (Torrance, CA) and ^{125}I as NaI was from Amersham. Iodo-Gen, 4-fluoro-3-nitrophenyl azide (FNPA), disuccinimidyl suberate (DSS), and *n*-octyl β-D-glucopyranoside (octyl glucoside) were purchased from Pierce. Anti-PTH-(1–34) antiserum was obtained from Meloy Laboratories (Springfield, VA). Reagents for NaDodSO$_4$/polyacrylamide gel electrophoresis were from Bethesda Research Laboratories. Staphylococcal protein A-Sepharose was purchased from Pharmacia, and Nonidet P-40 (NP-40) was obtained from LKB. Cell culture reagents were obtained from GIBCO.

Preparation of ^{125}I-Labeled [Nle]PTH-(1–34)-NH_2. All procedures were performed at room temperature unless otherwise specified. Iodo-Gen (100 μg) in methylene chloride was used to plate the inside of a 12 × 75 mm test tube according to the manufacturer's instructions. The iodination reaction was started by adding 10 μg of [Nle]PTH-(1–34)-NH_2 dissolved in 40 μl of 8 M urea and 2.0 mCi (1 Ci = 37 GBq) of ^{125}I. The tube was sealed and the reagents were allowed to react for 15 min with occasional agitation. The reaction was stopped by the addition of 0.3 ml of water and the mixture was drawn into a syringe containing anion-exchange resin (Bio-Rad AG 1-X8) for removing free ^{125}I. A filter was attached to the syringe and the unbound material was injected onto a 3.9 mm × 15 cm C_{18} HPLC column (Waters Nova Pak). The flow rate was 1 ml/min of 0.1% trifluoroacetic acid in 70% (vol/vol) water/30% acetonitrile with a linear gradient to 50% acetonitrile over 20 min. Column effluent was monitored for UV absorbance at 214 nm and for radioactivity in a flow-through isotope detector (Beckman), and 1-ml fractions were collected. Glycerol (10 μl) was added to the collected fractions containing the iodinated peptides and the samples were frozen and lyophilized.

Preparation of Photolabel. FNPA (25 μl of a 300 μM solution) in dimethyl sulfoxide was added to 100 μl of solution containing 1.5 μg of [Nle]PTH-(1–34)-NH_2 dissolved in 10 μM triethylamine in dimethyl sulfoxide. The solution was protected from light and agitated continuously for 4 hr. Glycine (15 μg) in 20 μl of 0.2 M sodium phosphate buffer (pH 7.4) was added to terminate the reaction. The product was used immediately for photolabeling studies.

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Abbreviations: PTH, parathyroid hormone; [Nle]PTH-(1–34)-NH_2, bovine [8-norleucine,18-norleucine,34-tyrosine]PTH-(1–34) amide; FNPA, 4-fluoro-3-nitrophenyl azide; DSS, disuccinimidyl suberate; NP-40, Nonidet P-40.

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Cell Culture. Rat osteosarcoma cells ROS 17/2.8 were obtained from Sevgi Rodan (5) and maintained in monolayer culture in modified Ham's F-12 medium (F-12) with 10% heat-inactivated fetal bovine serum and subcultured regularly with trypsin treatment before transfer. The monkey kidney cell lines COS and CV1, obtained from John Kopchick, were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum and subcultured routinely. All cell lines were free of mycoplasma contamination. Forty-eight hours prior to use the cells were plated into 100-mm culture dishes (Corning).

Covalent Attachment of Radiolabeled [Nle]PTH-(1–34)-NH_2 to Receptors. For photoaffinity labeling ^{125}I-[Nle]PTH-(1–34)-NH_2 conjugated to FNPA was added to intact cell monolayers in 2 ml of Hanks' balanced salt solution (HBSS) containing 1 mM $MgCl_2$ in the dark. [Nle]PTH-(1–34)-NH_2 (20 μg, 2 μM) was added to plates for competition experiments, for a ratio of unlabeled to radiolabeled ligand of 13:1. The plates were incubated for 20 min with occasional agitation and then exposed to a high-pressure mercury vapor lamp (Hanovia, Newark, NJ) for 5 min at a distance of 30 cm.

For the alternative method of chemical affinity crosslinking, DSS in dimethyl sulfoxide was added to a final concentration of 0.1 mM after incubation of ^{125}I-[Nle]PTH-(1–34)-NH_2 with intact cell monolayers for 20 min in HBSS containing 1 mM $MgCl_2$, and crosslinking proceeded for 5 min. The reaction was stopped by washing the plates three times with 5 ml of ice-cold buffer A (50 mM Tris·HCl/150 mM NaCl, pH 7.4).

Membrane Preparation. After covalent attachment of radiolabeled ligand, cell monolayers were washed two times with a buffer containing 50 mM Tris·HCl and 1 mM phenylmethylsulfonyl fluoride, pH 7.5 (buffer B), drained, and scraped into a 2-ml glass Dounce homogenizer with 1 ml of buffer B. Cells were disrupted with three pestle strokes and transferred to 12 × 75 mm glass culture tubes. Samples were spun at 800 × *g* for 1 min at 4°C and the supernatant was centrifuged at 13,100 × *g* in a tabletop Eppendorf centrifuge at 4°C to pellet membranes. Membrane pellets were either frozen and stored at −70°C or solubilized by stirring in buffer B containing 1% NP-40 at 4°C for 1 hr, then centrifugated at 100,000 × *g* for 1 hr at 4°C in a Beckman ultracentrifuge, model TL-100. Supernatants containing solubilized membrane proteins were stored at −70°C.

Preparation of IgG. To 50 ml of rabbit anti-PTH antiserum, 13.5 g of ammonium sulfate was added. The solution was mixed and allowed to stand at RT for 30 min, then centrifuged at 3000 × *g* for 30 min. The pellet was resuspended in 50 ml of water, 10.5 g of ammonium sulfate was added, and the precipitation procedure was repeated. The pellet was added to dialysis tubing with 25 ml of water and the solution was dialyzed against 2 liters of 0.07 M sodium phosphate buffer, pH 6.3 (buffer C), at 4°C. The dialysis buffer was changed twice over 48 hr. The dialysate was applied to a DEAE-cellulose anion-exchange column (Whatman DE52) in buffer C. IgG was eluted in the void volume of 7 ml.

Immunoprecipitation of Receptor–PTH Complex. A 100-μl sample of soluble membrane proteins was added to 400 μl of a dilution buffer containing 1% NP-40, 190 mM NaCl, 60 mM Tris·HCl at pH 7.4, 6 mM EDTA, and 10 units of aprotinin per ml. Fifty microliters of IgG or control antiserum was added, and the sample was incubated overnight at 4°C. Protein A-Sepharose beads (50 μl) in dilution buffer were added and the sample was mixed for 2 hr. After pelleting the Sepharose beads, the supernatant was aspirated and the beads were washed four times in wash buffer (0.1% NP-40/0.02% $NaDodSO_4$/150 mM NaCl/50 mM Tris·HCl, pH 7.5/5 mM EDTA/10 units of aprotinin per ml). A final wash of the beads using the above solution without detergent was performed and the supernatant was removed completely. The beads were boiled in electrophoresis sample buffer for 3 min and the supernatant was analyzed by $NaDodSO_4$ electrophoresis.

[Nle]PTH-(1–34)-NH_2-Sepharose Affinity Column. Eight milligrams of [Nle]PTH-(1–34)-NH_2 was allowed to react with 560 mg of CNBr-activated Sepharose 4B (Pharmacia) in 0.5 M NaCl/0.1 M $NaHCO_3$, pH 7.8 (buffer D), at 4°C for 24 hr. Essentially 100% of the peptide was coupled to the Sepharose as determined by the inclusion of a trace of iodinated [Nle]PTH-(1–34)-NH_2 in the reaction mixture. The [Nle]PTH-(1–34)-NH_2-conjugated Sepharose was washed extensively by alternate rinses in 0.1 M acetate buffer at pH 4.0 and buffer D at pH 8.0. A 0.2 M glycine solution in buffer D (pH 7.8) was added to the peptide-Sepharose and allowed to react at 4°C for 24 hr and the washing procedure was repeated. Prior to storage the column was washed with Dulbecco's phosphate-buffered saline (PBS).

Affinity Purification of IgG. Anti-PTH IgG (32.5 mg) was suspended in 10 ml of PBS with 0.2 M $MgCl_2$ and added to the PTH-Sepharose in a sealed vessel and mixed at 4°C for 24 hr. The mixture was poured into a glass column and washed with 5 vol of ice-cold PBS. One column volume of cold 10% (vol/vol) acetic acid was added to the resin mixture and the eluate was collected after 10 min. One column volume of PBS was applied to the column, collected, and combined with the acetic acid wash. After concentration on a YM30 filtration membrane (Amicon), the sample was applied to a Sephadex G-25 column and eluted with PBS at 4°C. The recovery of anti-PTH IgG was 4.6% (1.5 mg).

Preparation of IgG Affinity Column. The affinity-purified IgG was allowed to react with 280 mg of CNBr-activated Sepharose 4B by the above method. After the reaction had been quenched with 0.2 M glycine, the IgG-Sepharose was washed with 3 column volumes each of 10% (vol/vol) acetic acid and $NaHCO_3$ (pH 7.8), then 5 column volumes of PBS.

Immunoaffinity Separation of Receptor-PTH Complex. Membranes prepared from the monkey kidney COS cell line after photolabeling with ^{125}I-[Nle]PTH-(1–34)-NH_2 were solubilized as described above with 40 mM octyl glucoside and applied to the IgG-Sepharose column. The column was sealed and rotated at 4°C for 24 hr, then washed with 5 column volumes of PBS and 1 volume each of the following in sequence: 50 μM [Nle]PTH-(1–34)-NH_2, 3 M NaCl, 1 M acetic acid. Eluates were collected and their radioactivities were measured in a gamma counter, and samples were prepared for $NaDodSO_4$ electrophoresis.

$NaDodSO_4$ Gel Electrophoresis. Slab gels (10 × 15 × 0.15 cm) were prepared by the method of Laemmli (6) under reducing conditions unless otherwise specified. Equal cpm were added per lane except as indicated in figure legends. Molecular weight marker proteins were run in separate lanes. Electrophoresis was at constant current (4 mA per gel) overnight or until the bromophenol blue dye front reached the bottom. After electrophoresis the gel was stained with Coomassie blue in 7% acetic acid/50% methanol and destained in the same solution, then dried and mounted for autoradiography on Kodak SB-5 x-ray film using DuPont Cronex intensifying screens.

Protein Determination. Protein was determined by using the method of Bradford (7) with bovine serum albumin as the standard.

RESULTS

Purification of Radioligand. ^{125}I-[Nle]PTH-(1–34)-NH_2 was purified by HPLC prior to use in cell labeling studies. Using this method, we separated unlabeled and mono- and diiodinated forms of the peptide. The retention times were 9.7 min for noniodinated [Nle]PTH-(1–34)-NH_2, 10.6 min for monoiodinated [Nle]PTH-(1–34)-NH_2, and 11.5 min for diiodinated [Nle]PTH-(1–34)-NH_2. The HPLC elution profile (A_{214}) is

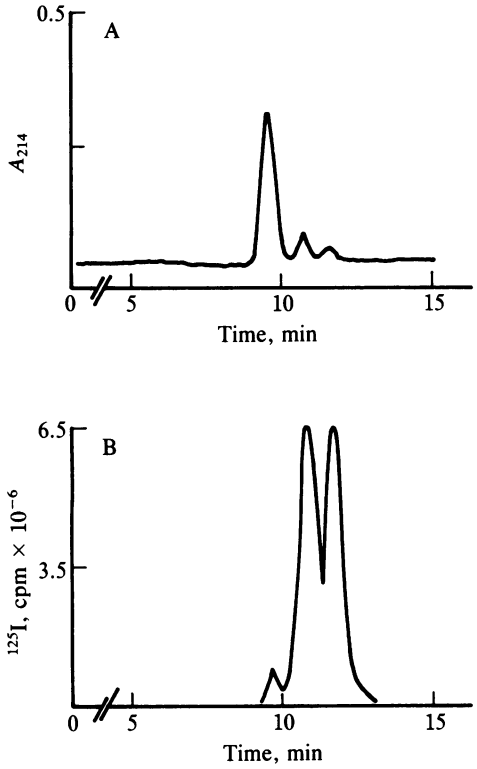

FIG. 1. Purification of ^{125}I-[Nle]PTH-(1–34)-NH_2 by HPLC. (*A*) A_{214} elution profile of noniodinated, monoiodinated, and diiodinated [Nle]PTH-(1–34)-NH_2. (*B*) Elution profile obtained by using a flow-through isotope detector. Specific activity of pooled monoiodinated and diiodinated radioligand was 600 mCi/mg.

shown in Fig. 1*A*. The elution profile generated by using the Beckman flow-through isotope detector is shown in Fig. 1*B*. Mono- and diiodinated fractions were pooled for use in labeling studies. The total yield of radiolabeled analogs was approximately 1.5 μg (366 pmol), based on area under the A_{214} peaks of the mono- and diiodinated peptides. The radioligand had a specific activity of 600 mCi/mg and contained, on average, 1.2 atoms of iodine per molecule of [Nle]PTH-(1–34)-NH_2.

Covalent Labeling of PTH Receptors. Both photoaffinity labeling and chemical (affinity) crosslinking were used. Photoaffinity and chemical crosslinking of ^{125}I-[Nle]PTH-(1–34)-NH_2 to intact rat osteosarcoma cells are compared in Fig. 2, which shows an autoradiograph of samples generated by both labeling techniques. Prominent bands are seen at 95 kDa, 69–70 kDa (a doublet), and 28 kDa in Fig. 2*A*; these bands decrease in intensity when cells are exposed to excess competing unlabeled hormone. When the affinity crosslinking approach is used, a similar pattern of protein banding is observed, although the 70-kDa band does not clearly appear as a doublet with this technique.

In solubilized membranes from photolabeled monkey kidney CV1 cells, the 28-kDa component is seen in the presence (Fig. 2*C*, lanes 1 and 2) but not the absence (Fig. 2*C*, lanes 3 and 4) of 2-mercaptoethanol, suggesting that NP-40-solubilized PTH receptors contain subunits associated by disulfide bridges. Alternatively, this lower molecular weight band may have been generated by proteolysis of the PTH receptor. However, this band is observed in the presence of the following protease inhibitors: phenylmethylsulfonyl fluoride at 1 mM, aprotinin at 10 units/ml, pepstatin at 10 μg/ml, leupeptin at 10 μg/ml, EDTA at 2 mM, soybean trypsin inhibitor at 100 μg/ml (data not shown).

Immunoprecipitation of the Receptor-PTH Complex. The autoradiograph in Fig. 3 shows competable labeling of 95-, 70-, and 28-kDa membrane components (lanes 1 and 2) in samples of proteins solubilized with NP-40 after crosslinking of ^{125}I-[Nle]PTH-(1–34)-NH_2 to ROS 17/2.8 cells with DSS. After immunoprecipitation, 70- and 28-kDa bands are visible (lanes 3 and 4); the 95-kDa band was not observed after precipitation under these conditions. Approximately 30% of the radiolabeled material was immunoprecipitated with an optimal concentration of anti-PTH IgG.

Due to the large quantity of bovine serum albumin in fetal calf serum used for *in vitro* cultivation of cell lines, we included an antibody to bovine serum albumin (Cappel Laboratories, Cochranville, PA) as an additional control for the immunoprecipitation experiments. No radiolabeled bands were visible when anti-albumin was used (Fig. 3, lane

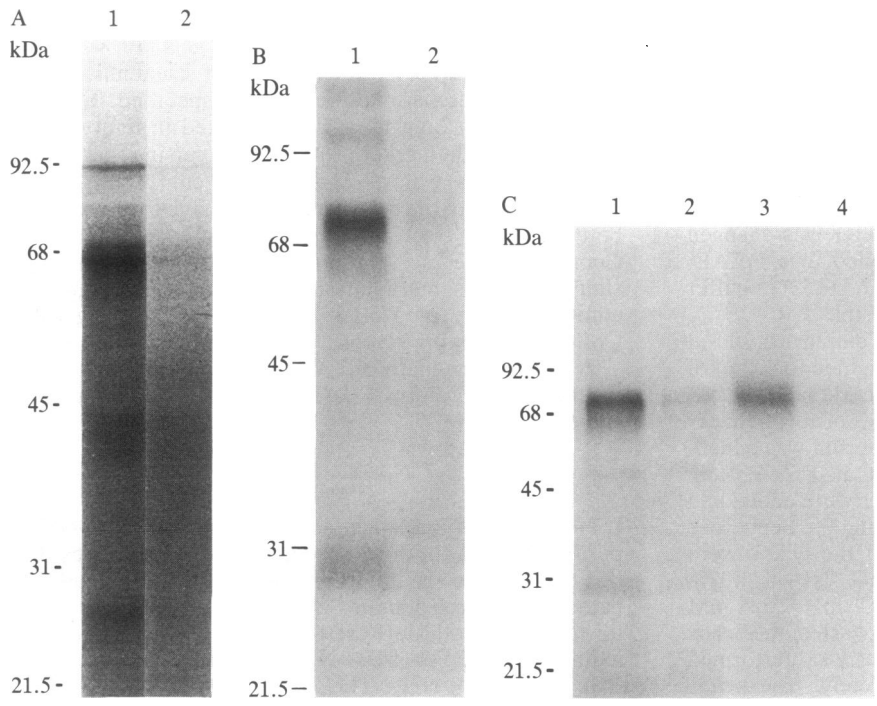

FIG. 2. Autoradiographs after NaDodSO$_4$/polyacrylamide gel electrophoresis of photoaffinity- and affinity-labeled (crosslinked) PTH receptors. (*A*) Photoaffinity labeling of ROS 17/2.8 cells with FNPA-^{125}I-[Nle]PTH-(1–34)-NH_2. Lane 1, photolabile radioligand was added in the absence of competing hormone. Lane 2, photolabeling was performed in the presence of 20 μg of [Nle]PTH-(1–34)-NH_2. (*B*) Affinity labeling (crosslinking) of ROS 17/2.8 cells with DSS. Lane 1, labeling with ^{125}I-[Nle]PTH-(1–34)-NH_2 was done in the absence of competing hormone. Lane 2, labeling in the presence of competing hormone. (*C*) Photoaffinity-labeled monkey kidney CV1 cells prepared for electrophoresis under reducing conditions, in the absence (lane 1) and presence (lane 2) of excess [Nle]PTH-(1–34)-NH_2. Identical samples were prepared under nonreducing conditions in the absence (lane 3) and presence (lane 4) of excess [Nle]PTH-(1–34)-NH_2.

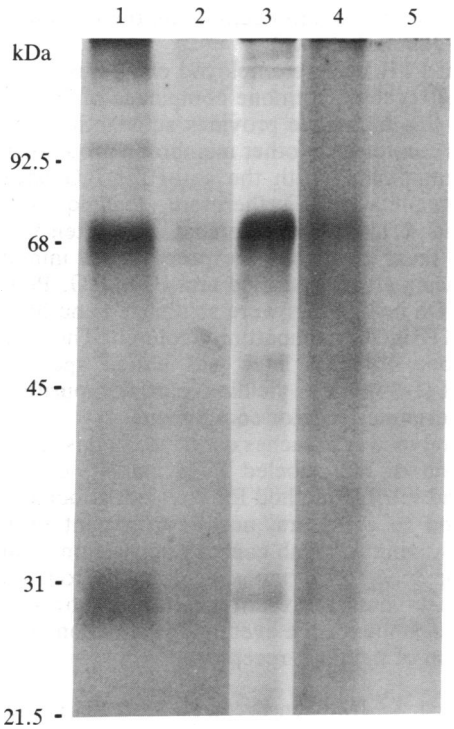

FIG. 3. Immunoprecipitation of ^{125}I-[Nle]PTH-(1–34)-NH_2-receptor complex. The radioligand was covalently attached to ROS 17/2.8 cells by using DSS in the absence (lanes 1, 3, and 5) or presence (lanes 2 and 4) of excess [Nle]PTH-(1–34)-NH_2. After solubilization, either samples were prepared directly for electrophoresis (lanes 1 and 2) or aliquots containing equal amounts of protein were incubated overnight with anti-PTH IgG (lane 3 sample, 300,000 cpm; lane 4 sample, 350,000 cpm) or antibody to bovine serum albumin (lane 5 sample, 300,000 cpm). Equal cpm were added to lanes 1 and 2. Total cpm obtained after immunoprecipitation were added to lanes 3 (100,000 cpm), 4 (25,000 cpm), and 5 (4200 cpm). The gels were dried and exposed to Kodak SB-5 x-ray film. An autoradiograph of the gel is shown.

5). In addition, no bands were visible when nonimmune rabbit serum was used (data not shown). Coomassie blue staining of gels prior to autoradiography failed to show any staining bands in the approximate molecular weight region of the receptor, suggesting that the quantities of peptide detected by autoradiography in this experiment are too small to be detected by this dye.

Receptor-PTH Complex Binding to an Immunoaffinity Column. In an effort to improve the effectiveness of the anti-ligand antibody technique, anti-PTH IgG was affinity purified by passage over a PTH-Sepharose column and then attached to a Sepharose column.

Solubilized photolabeled COS membranes were placed on the column. As shown in Fig. 4, elution of the column with 50 μM [Nle]PTH-(1–34)-NH_2 released the 70- and 28-kDa membrane components (lane 1). Further elution with 3 M NaCl failed to release any radiolabeled membrane components (lane 2). However, subsequent washing of the column with 1 M acetic acid released a 70-kDa membrane component. The [Nle]PTH-(1–34)-NH_2 and acetic acid washes eluted 44% and 41% of the bound ^{125}I-labeled components, respectively.

DISCUSSION

The parathyroid hormone receptor (or a binding subunit of the receptor) has been identified in previous studies using photoaffinity labeling techniques (2–4). The hormone-receptor complex was reported to have an apparent molecular

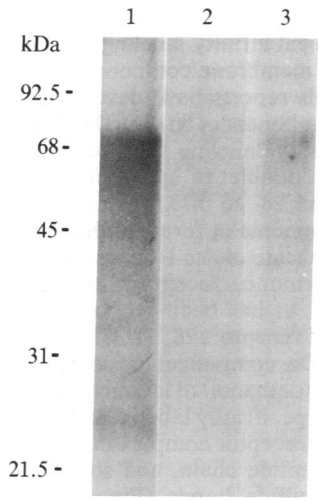

FIG. 4. Autoradiograph of eluates from immunoaffinity column. Affinity-purified IgG was attached to a CNBr-activated Sepharose column, and solubilized photolabeled COS membranes were applied. After extensive washing, the column was incubated with 50 μM [Nle]PTH-(1–34)-NH_2 (lane 1), followed by 3 M NaCl (lane 2), then 1 M acetic acid (lane 3). Initial washes and eluates were prepared for electrophoresis. Column washes prior to elution with [Nle]PTH-(1–34)-NH_2 appeared to be devoid of radiolabeled hormone-receptor complex (data not shown). The [Nle]PTH-(1–34)-NH_2 and acetic acid washes eluted 44% and 41% of the bound ^{125}I-labeled components, respectively. The 3 M NaCl failed to release any radiolabeled membrane components.

mass of 70 kDa (2–4). Furthermore, a membrane component of identical apparent molecular mass (as determined by $NaDodSO_4$ gel electrophoresis) was found in several PTH-responsive tissues from dogs and humans, indicating that the physicochemical properties of the PTH receptor have been highly conserved during evolution (4), as has the sequence of PTH (1). This initial characterization of the receptor has also served to guide further efforts directed at harvesting and isolating the PTH receptor.

Progress in this area has been hampered by the small number of PTH receptors present in target tissues and the difficulty of obtaining a biologically active radiolabeled form of PTH that can be used reproducibly for covalent attachment to the receptor. Hence, our recent research efforts have focused on obtaining a purified radiolabeled analog of PTH, devising a reliable method for covalently attaching this radioligand to the receptor, and developing a potentially selective and high-capacity technique for separating the PTH receptor from other membrane constituents.

Purification of the products of iodination of [Nle]PTH-(1–34)-NH_2 by HPLC permitted the separation of uniodinated peptide from the mono- and diiodinated forms (Fig. 1). We can now routinely prepare radioligand of estimated specific activity of 600 mCi/mg for use in photoaffinity labeling and chemical crosslinking studies.

In anticipation of increasing our scale of receptor labeling for eventual receptor isolation, we sought to simplify this procedure for covalent attachment of the ligand to the receptor. Chemical affinity crosslinking appeared to be a promising approach because it avoids the difficulties of working with large quantities of radioactivity and cells in the dark. Using the rat osteosarcoma line, we have demonstrated that the bifunctional chemical crosslinking agent DSS labels a mixture of membrane components closely similar to those identified by the photoaffinity labeling approach, although a doublet at the 68- to 70-kDa region is not clearly observed with this technique.

An additional observation made by using both the photoaffinity and chemical affinity labeling approaches is the presence of several membrane components that are specifically labeled. Previous reports have described the receptor-PTH complex as a component of 60–70 kDa (2–4). In these studies, using photoaffinity labeling techniques, we observed the appearance of a doublet of apparent molecular mass 68–70 kDa and 95- and 28- to 30-kDa bands that may represent additional components or forms of the receptor. In addition, the high-affinity state of the PTH receptor may be a ternary complex of hormone, receptor, and guanine nucleotide-binding protein, as has been proposed for other adenylate cyclase-coupled receptors (8, 9). Under nonreducing conditions, the 28-kDa component is poorly visible. Reduction (using 2-mercaptoethanol) of identical samples resulted in the generation of a specifically labeled 28-kDa band. This finding suggests a PTH-receptor complex may be composed of more than one polypeptide chain, and some chains may be held together by disulfide bonds (Fig. 2*C*). Alternatively, the 28-kDa band may be derived from proteolytic cleavage of a higher molecular weight receptor component. However, the addition of several protease inhibitors during manipulation of cell lysates (after covalent attachment of ^{125}I-[Nle]PTH-(1–34)-NH_2 to the receptor) failed to prevent the appearance of the 28-kDa band on the autoradiographs.

In an effort to develop a highly resolving, potentially high-capacity technique for separating receptor from other membrane constituents, we explored the feasibility of using immunoaffinity methods. Several laboratories have employed anti-ligand antisera in purification of membrane proteins (10, 11), including hormone receptors (12–14). This approach is especially useful in the early stages of devising an overall scheme for receptor purification, when antibodies to the receptor are not yet available because sufficient quantities of receptor have not been harvested for immunization purposes. In addition, this approach circumvents the need to monitor hormone binding to the receptor throughout the purification scheme. This latter issue may be an important consideration for isolation of the PTH receptor because functionality (as determined by avidity for hormone) may be greatly diminished after solubilization. Although Malbon and Zull reported the binding of native bovine PTH-(1–84) to Triton X-100-solubilized bovine renal membranes (15), Nissenson *et al.* (16) recently were able to obtain soluble PTH-receptor complexes only if the agonist was added prior to solubilization.

Prerequisite to using ligand-binding immunoaffinity approaches for purification of receptor-hormone complexes is the availability of an antiserum that recognizes hormone in its covalent receptor-bound conformation—i.e., the structural determinants within the hormone molecule that are necessary for binding to the immunoglobulin must be accessible even during receptor interaction. Seven commercially available anti-PTH-(1–34) antisera were evaluated. Of these, only two were capable of immunoprecipitating the receptor-hormone complex (data not shown).

The anti-PTH IgG we employed can be used to immunoprecipitate receptor-hormone complexes of 70 and 28 kDa. Although this technique provides substantial separation of the PTH receptor from other membrane proteins, the receptor is contaminated with the anti-PTH IgG added during immunoprecipitation. Furthermore, techniques for reutilizing valuable IgG are cumbersome and often wasteful. To eliminate these problems, we prepared an immunoaffinity column using affinity-purified anti-PTH IgG. Photoaffinity-labeled COS cell lysates were solubilized and found to bind to anti-PTH IgG immunoaffinity column. The bound receptor-hormone complex later was eluted specifically with [Nle]PTH-(1–34)-NH_2, yielding partially purified 70- and 28-kDa hormone-receptor components.

The combined approaches described in this report, namely purification of radiolabeled PTH analog, utilization of a simple and reliable method for covalent attachment of PTH radioligand to receptors, and development of potentially highly resolving and high-capacity separation techniques for separating receptor-hormone complexes from other membrane constituents, represent critical steps toward devising a practical scheme for the eventual purification and structural elucidation of the PTH receptor.

1. Potts, J. T., Jr., Kronenberg, H. M. & Rosenblatt, M. (1982) *Adv. Protein Chem.* **33,** 323–396.
2. Coltrera, M. D., Potts, J. T., Jr., & Rosenblatt, M. (1981) *J. Biol. Chem.* **256,** 10555–10559.
3. Draper, M. W., Nissenson, R. A., Winer, J., Ramachandran, J. & Arnaud, C. D. (1982) *J. Biol. Chem.* **257,** 3714–3718.
4. Goldring, S. R., Tyler, G. A., Krane, S. M., Potts, J. T., Jr., & Rosenblatt, M. (1984) *Biochemistry* **23,** 498–502.
5. Majeska, R. J., Rodan, S. B. & Rodan, G. A. (1980) *Endocrinology* **107,** 1494–1503.
6. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
7. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
8. DeLean, A., Stadel, J. M. & Lefkowitz, R. J. (1980) *J. Biol. Chem.* **255,** 7108–7117.
9. Lefkowitz, R. J., Caron, M. G. & Stiles, G. L. (1984) *N. Engl. J. Med.* **310,** 1570–1579.
10. Wada, H. G., Hass, P. E. & Susman, H. H. (1979) *J. Biol. Chem.* **254,** 12629–12635.
11. Proia, R. L., Hart, D. A., Holmes, R. K., Holmes, K. V. & Eidels, L. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 685–689.
12. Heinrich, J., Pilch, P. F. & Czech, M. P. (1980) *J. Biol. Chem.* **255,** 1732–1737.
13. Metsikko, K. & Rajaniemi, H. (1980) *Biochem. Biophys. Res. Commun.* **95,** 1730–1736.
14. Simonds, W. F., Burke, T. R., Jr., Rice, K. C., Jacobson, A. E. & Klee, W. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4974–4978.
15. Malbon, C. C. & Zull, J. E. (1975) *Biochem. Biophys. Res. Commun.* **66,** 179–187.
16. Nissenson, R. A., Mann, E., Winer, J., Teitelbaum, A. P. & Arnaud, C. D. (1986) *Endocrinology* **118,** 932–939.