Photoaffinity Labeling of Parathyroid Hormone Receptors in Clonal Rat Osteosarcoma Cells*

(Received for publication, August 31, 1987)

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A photoactive derivative of a sulfur-free bovine parathyroid hormone (PTH) analogue, [Nle\(^{a},N^{a},(4\text{-azido}-2\text{-nitrophenyl})\text{Lys}^{18},\text{Nle}^{18},\text{Tyr}^{34}]\text{bovine PTH-(1\text{-34})-NH}_{2}\) (NAP-NlePTH), was purified from the products of the reaction of [Nle\(^{a},N^{a},\text{Tyr}^{34}]\text{bovine PTH-(1\text{-34})-NH}_{2}\) (NlePTH) with 4-fluoro-3-nitrophenylazide and was used to identify binding components of the PTH receptor in clonal rat osteosarcoma cells (ROS 17/2.8). The purified analogue, NAP-NlePTH, is a fully active agonist in three different ROS 17/2.8 cell bioassays: 1) specific binding to saturable PTH receptors; 2) stimulation of cyclic AMP accumulation; and 3) inhibition of cellular alkaline phosphatase activity; this analogue gave dose-response curves parallel to and 25-33% as potent as its parent molecule, NlePTH. Radioiodinated NAP-NlePTH (\(^{125}\text{I}\)-labeled NAP-NlePTH) retained maximal receptor-binding potency. Radioligand saturation studies in intact cells showed that the \(K_{D}\) of PTH receptors for the photoligand was slightly less than that for \(^{125}\text{I}\)-labeled NlePTH (2.8 and 0.8 nM, respectively), but that the \(B_{max}\) was essentially identical for both radioligands (8 fmol/10\(^6\) cells). Photoaffinity labeling of ROS 17/2.8 cells revealed several \(^{125}\text{I}\)-labeled macromolecular components by sodium dodecyl sulfate-polyacrylamide gel electrophoreses. One predominant \(^{125}\text{I}\)-labeled band, having an apparent \(M_{r}\) of 80,000 daltons (including \(M_{r} = 4,347\) ligand; hereafter referred to as the \(M_{r} = 80,000\) protein), was consistently demonstrated in both reducing and nonreducing conditions. Its labeling was completely inhibited by coincubation with NlePTH (10 nM) at 26-fold molar excess to the photoligand, but not by biologically inactive PTH fragments or unrelated hormone. Labeling of several other macromolecular components persisted in the presence of NlePTH (1 \(\mu\)M). Only the labeling of the \(M_{r} = 80,000\) protein showed kinetics for photoaffinity labeling; the dose of \(^{125}\text{I}\)-labeled NAP-NlePTH (0.8 nM) to half-saturate labeling of the \(M_{r} = 80,000\) protein was close to the \(K_{D}\) (2.8 nM) of specific binding of the photoligand to receptors in intact ROS 17/2.8 cells. Pretreatment of the cells with NlePTH and dexamethasone led to the predicted proportional decrease or increase, respectively, in labeling of the \(M_{r} = 80,000\) protein. Our data, using a highly purified photactive derivative of PTH, having carefully defined chemical and biological properties, show a plasma membrane component of \(M_{r} = 80,000\) in ROS 17/2.8 cells that possesses the affinity, binding capacity, and physiological characteristics of the PTH receptor.

The physiological action of parathyroid hormone (PTH)\(^{1}\) in its target tissues (1-15) is believed to be initiated by the interaction of the hormone with specific, high-affinity (\(K_{D}\) values = \(10^{-10}-10^{-8}\)), low-capacity receptors in the plasma membrane (5-7, 11-13). Different techniques have been employed to identify and physicochemically characterize the hormone binding component of PTH receptors in several biological systems (15-19). These studies have illustrated the potential of the techniques, but the data have been inconsistent, showing PTH receptors of different apparent sizes, and, in some reports, multiple hormone binding components. Although analyses have used different receptor sources in some instances, these differences appear unlikely to account for the discrepant results.

The structural information concerning PTH receptors was initially provided by photoaffinity labeling technique (15, 16). Earlier studies, using three different nitroarylazide photo-probes, suggested that the binding component of the PTH receptor is a monomeric protein of \(M_{r} = 70,000\) daltons (15, 17) or \(M_{r} = 60,000\) daltons (16) in canine renal membrane, and \(M_{r} = 70,000\) daltons in human fibroblasts and cultured cells of human skeletal tumor origin (17). More recently, Wright et al. (18) suggested the presence of multiple hormone binding components of the PTH receptor (\(M_{r} = 95,000, 70,000,\) and 28,000) in osteoblast-like clonal rat osteosarcoma cells (ROS 17/2.8) and monkey kidney cell lines (COS and CV1) using both photoaffinity-labeling and chemical cross-linking techniques. In addition, Nissenson et al. (19) reported separate hormone binding components of \(M_{r} = 130,000, 85,000,\) and 55,000 in canine renal membrane by chemical cross-linking.

We reasoned that more rigorous control of the experimental techniques might resolve some of these apparent discrepancies. Accordingly, the current studies differ from those reported by our laboratory (15, 17) and others (16, 18, 19) in several major respects. We first synthesized, purified to homogeneity, and chemically characterized the most biologically active component from the multiple photodervitized prod-

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\(^{1}\) The abbreviations used are: PTH, parathyroid hormone; ROS, rat osteosarcoma cells; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoreses; BSA, bovine serum albumin; DTT, dithiothreitol; FNPA, 4-fluoro-3-nitrophenylazide; NlePTH, [Nle\(^{a},N^{a}\), Tyra\(^{34}\)bovine PTH-(1\text{-34})-NH\(_{2}\) NAP-NlePTH, [Nle\(^{a},N^{a},(4\text{-azido}-2\text{-nitrophenyl})\text{Lys}^{18},\text{Nle}^{18},\text{Tyr}^{34}]\text{bovine PTH-(1\text{-34})-NH}_{2}\) PHS, phosphate-buffered saline; HPLC, high performance liquid chromatography; IBMX, 3-isobutyl-1-methylxanthine; HBSS, Hank’s balanced salt solution; PNP, para-nitrophenol; PNPP, disodium para-nitrophenylphosphate; TEA, triethylamine.
ucts arising from the reaction of the PTH analogue, [Nle\(^6\),Nle\(^8\),Tyr\(^{14}\)]bovine PTH-(1–34)-NH\(_2\) (NlePTH), with 4-fluoro-3-nitrophenylazide (FNPA).\(^2\) We hoped that photo-labeling of PTH receptors using this purified photophobe which we found to be selectively derivatized at Lys\(^{15}\) of the sequence, [Nle\(^6\),N-e-(4-azido-2-nitrophenyl)Lys\(^{15}\),Nle\(^8\),Tyr\(^{14}\)] bovine PTH-(1–34)-NH\(_2\) (NAP-NlePTH), might provide more specific identification of PTH receptors than could be achieved using a heterogeneous mixture of variably derivatized synthetic fragments. Second, we rigorously assessed the biological activity of NAP-NlePTH in several assay systems and characterized the specificity and binding properties of this derivative to ROS 17/28 cell PTH receptors. Lastly, we conducted our experiments at 15 °C to minimize potential proteolytic degradation and internalization of the photoligand-receptor complex.

In the present study, we have demonstrated that NAP-NlePTH possesses full agonist activity in three different intact ROS 17/2.8 cell bioassays and utilized \(^{125}\)I-labeled NAP-NlePTH as a high-affinity photoaffinity label to physicochemically identify the binding component(s) of PTH receptors in ROS 17/2.8 cells. While we confirm the existence of multiple acceptor macromolecules for the PTH photoaffinity label similar to those reported by others (18, 19), only one protein of an approximate \(M_\text{r} = 80,000\) exhibits the affinity, binding capacity, and physiological characteristics of the PTH receptor as determined using radiolabeled, underivatized NlePTH.

**MATERIALS AND METHODS\(^3\)**

**RESULTS**

**Photoaffinity Labeling of PTH Receptors in ROS 17/2.8 Cells**—ROS 17/2.8 cells were incubated (4 h, 15 °C) with radioiodinated NAP-NlePTH in the dark, photolyzed, extensively rinsed to remove unbound radioligand, and solubilized with SDS-PAGE buffer. The samples then were analyzed by autoradiography after SDS-PAGE. Photolysis of ROS 17/2.8 cells after incubation with radioiodinated NAP-NlePTH resulted in covalent attachment of the radioligand to several macromolecules of different sizes (Fig. 6). The predominant labeled component consistently was a diffuse band of an apparent \(M_\text{r} = 80,000\) daltons, whose electrophoretic mobility was unaltered by addition of DTT (100 mM) at the time of SDS solubilization (Fig. 6, lanes 1 and 2). Therefore, subsequent SDS-PAGE analyses were performed in the presence of DTT (100 mM). Coincubation of the cells with varying doses of NlePTH resulted in dose-dependent, selective inhibition of the labeling of the \(M_\text{r} = 80,000\) protein. Labeling of the \(M_\text{r} = 80,000\) protein by \(^{125}\)I-labeled NAP-NlePTH (0.4 nM) was partially inhibited by NlePTH (0.1 nM) and abolished by 25-fold molar excess (10 nM) or higher of NlePTH (Fig. 6, lanes 3–5). Photolabeling of the \(M_\text{r} = 80,000\) protein was inhibited only by biologically active PTH peptide: neither bovine PTH (53–84) nor human ACTH (1–39) (1 μM, both) affected labeling of the \(M_\text{r} = 80,000\) protein (Fig. 6, lanes 6 and 7). Photolabeling of the \(M_\text{r} = 80,000\) protein was dependent on both the duration of incubation with \(^{125}\)I-labeled NAP-NlePTH and on the length of UV irradiation (data not shown): labeling of the \(M_\text{r} = 80,000\) protein was apparent

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\(^3\) Portions of this paper (including “Materials and Methods,” part of “Results,” Table I, and Figs. 1–5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

**Fig. 6.** Photoaffinity labeling of PTH receptors on ROS 17/2.8 cells. Confluent cells were incubated in the dark (4 h, 15 °C) with \(^{125}\)I-labeled NAP-NlePTH (0.4 nM) in the absence (lanes 1 and 2) or presence (lanes 3–7) of various doses of competing ligands, or after pretreatment for 1, 2, or 3 days with various doses of peptide hormones (lanes 8–17), as indicated below each lane. After photolysis, cells were rinsed and solubilized in SDS-PAGE sample buffer containing DTT (100 mM) except for the sample in lane 1 which was solubilized with SDS-PAGE sample buffer without DTT. SDS-denatured samples were applied onto a 5–20% SDS-PAGE. After electrophoresis, the gels were stained, destained, and dried, followed by autoradiography (2 days, −80 °C), as described under “Materials and Methods.” The \(M_\text{r}\) standards (×10\(^3\)) are indicated.

**Fig. 7.** Time course of photoaffinity labeling of PTH receptors. Confluent cells were incubated in the dark (4 h, 15 °C) with \(^{125}\)I-labeled NAP-NlePTH (0.5 nM) for varying periods of time (0.5–5 h) in the absence (odd-numbered lanes) or presence (even-numbered lanes) of NlePTH (10 nM). After photolysis, cells were rinsed and solubilized with SDS-PAGE sample buffer. SDS-denatured samples were applied onto a 9–13% SDS-PAGE, and autoradiography (2 days, −80 °C) was performed, as described under “Materials and Methods.” The \(M_\text{r}\) standards (×10\(^3\)) are indicated.

within 30 min of incubation, was maximal by 4 h (Fig. 7), and was stable for at least an additional 2 h (data not shown). Neither incubation of the cells (4 h, 15 °C) with prephotolyzed \(^{125}\)I-labeled NAP-NlePTH (30 min, 4 °C at a source object distance of 5 cm) followed by photolysis (data not shown) nor incubation with \(^{125}\)I-labeled NAP-NlePTH in the dark without subsequent UV irradiation (29) resulted in labeling of the \(M_\text{r} = 80,000\) protein.

The photochemical labeling of the \(M_\text{r} = 80,000\) protein was saturable (Fig. 8). Photolysis of the cells after incubation with incremental doses of \(^{125}\)I-labeled NAP-NlePTH (2200 Ci/mmol, 0.78–32.8 nM) in the absence (lanes 4–11) and presence (lanes 1–3) of NlePTH (1 μM) showed that labeling of the \(M_\text{r} = 80,000\) protein was saturated at 3.2 nM or higher doses of the photoligand. Direct quantitation of the radioactivity in the \(M_\text{r} = 80,000\) bands from the dried gel revealed that the
half-maximally effective ligand concentration was around 0.8 nM (Fig. 8B), closely similar to the dissociation constant ($K_d = 2.8$ nM) obtained from radioligand saturation studies (Fig. 4). In sharp contrast, labeling of the other macromolecular components (including $M_r = 260,000$, $190,000$, $160,000$, $120,000$, $88,000$, $70,000$, $53,000$, $42,000$, $35,000$, $29,000$, and $22,000$) was not consistently observed, did not follow saturation kinetics, and was not completely inhibited by coinucubation with NlePTH (1 μM) (Figs. 6–8). Labeling efficiency of the $M_r = 80,000$ protein was $8.8 \pm 0.9\%$ (mean $\pm$ S.D., $n = 4$) of the total bound $^{[125]}$I-labeled NAP-NlePTH ($<1.4$ nM) as determined by direct measurement: of the radioactivity in slices of the dried gels.

We have previously used radioligand saturation analysis to show that prolonged exposure of ROS 17/2 and 17/2.8 cells to PTH agonists (but not PTH antagonists) and glucocorticoids results in a time- and dose-dependent decrease (13, 31) and increase (13), respectively, of the available receptor number ($B_{max}$) without a change in binding affinity ($K_d$). These alterations in $B_{max}$ are associated with concomitant changes in PTH-stimulable adenylate cyclase (30, 31). Therefore, we examined the effects of NlePTH and dexamethasone on covalent photoligand labeling of the $M_r = 80,000$ protein. ROS 17/2.8 cells were treated (1 to 3 days) with NlePTH (10$^{-11}$–10$^{-7}$ M). After photoaffinity labeling, the cells were solubilized in SDS-PAGE sample buffer and analyzed by SDS-PAGE. Prolonged treatment of cells with NlePTH selectively reduced the extent of $M_r = 80,000$ protein labeling without changing the electrophoretic mobility of the labeled complexes (Fig. 6). The decreased labeling of the $M_r = 80,000$ protein was dependent on both duration of pretreatment (Fig. 6, lanes 2, and 8–10) and the dose of NlePTH (Fig. 6, lanes 2, and 11–16). Pretreatment (3 days) of cells with NlePTH (10$^{-7}$ M) maximally decreased photolabeling of the $M_r = 80,000$ protein to 20% of control, based on densitometric analysis of the autoradiogram (Fig. 6, lanes 2–16). This is identical with the maximal PTH-induced decrease in available receptor number (10 to 20% of control levels) as determined by saturation analysis using $^{[125]}$I-labeled NlePTH (31).

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ROS cells macromolecules (Fig. 6, lane 17). Confuent cells then were treated with dexamethasone (10^{-10}–10^{-7} M) for 2 days, and after photoaffinity labeling, the SDS-denatured samples were analyzed by SDS-PAGE. Dexamethasone (10^{-7} M) increased photolabeling of the $M_0 = 80,000$ protein by 1.8-fold (as assessed by densitometric analysis of the autoradiogram) (Fig. 9A) which was completely inhibited by coincubation with NlePTH (10 nM). The dexamethasone effects on photolabeling of the $M_0 = 80,000$ protein were dose-dependent (Fig. 9B), with increases apparent at a dose as small as 10^{-10} M (Fig. 9B, lane 3). This stimulatory effect by dexamethasone on photoaffinity labeling of the $M_0 = 80,000$ protein in ROS 17/2.8 cells was correlated quantitatively, with the increased specific binding of 125I-labeled NlePTH to cells treated with dexamethasone (10^{-7} M), 15.0% ± 0.5% compared to control cells (8.7% ± 0.2%) (mean ± S.D., n = 4, p < 0.001). Cell number, protein concentration, and protein staining pattern were indistinguishable in control and cells treated with either PTH or dexamethasone (data not shown). Thus, these data support our conclusion that the $M_0 = 80,000$ protein represents the only component of ROS 17/2.8 cells possessing the affinity, binding capacity, and physiochemical properties of PTH receptors.

**DISCUSSION**

We and others (11, 15, 14) have shown that the specific, high-affinity, low-capacity binding of radiodinated bovine PTH-(1–84) or [Nle8,Nle18,Tyr34]bovine PTH-(1–34)-NH$_2$ to a single class of binding sites in ROS 17/2.8 cells represents binding of the hormone to its physiological receptor. The approaches we have used in these studies appear to have provided more satisfactory data than some of our earlier attempts (15, 17), or those of other laboratories (18), to define the physicochemical properties of the PTH receptor. Purifying one of the nitrophenylamide derivatives of NlePTH to homogeneity, an analogue in which the photoreactive group is coupled exclusively onto Lys16, has enabled us to characterize the biologic properties of this photoprobe and then use this homogeneous photoligand to study the properties of the PTH receptor. We have shown that NAP-NlePTH is a fully active agonist in three different bioassay systems, with 25–33% of the potency of the parent molecule, NlePTH. When this analogue is radiodinated, purified by HPLC, and reacted with PTH receptors in the dark, its binding properties are nearly identical with those of radiodinated, but not photodervatized, NlePTH; specifically, 1) it binds to the same number of receptor sites with only slightly lower affinity, 2) binding is to sites that are totally displaced only by biologically active PTH agonists and antagonist, and 3) the photoprobe binds with an essentially identical association rate. It is interesting to note that the apparent $K_d$ values observed in competition studies using the same unlabeled ligands and 125I-labeled NAP-NlePTH are slightly shifted to the lower range (Fig. 5), compared to those using 125I-labeled NlePTH (Fig. 1A), a finding expected from the lower binding affinity of the radiodinated photoligand.

Characteristics of the photolabeling of the $M_0 = 80,000$ protein are closely similar to those observed of binding of the photoprobe to intact ROS cells in the dark (i.e. without photoactivation): labeling of PTH receptors by the photoligand is saturable with a half-maximal dose of 0.8 nM and is totally displaced by coincubation with 26-fold excess of competing NlePTH (10 nM). Moreover, there is an excellent correlation between the magnitude of photoaffinity labeling of the $M_0 = 80,000$ protein and that of hormone-induced alterations in the number of apparent availability of PTH receptors by treatment with PTH (31) and glucocorticoids as determined by 125I-labeled NlePTH.

It is not clear why our data differ from those of Wright et al. (18) and Nissenson et al. (19), which suggested the presence of specifically labeled 95-, 70-, and 28-kDa binding components in ROS 17/2.8 cells (18), and 130-, 85-, and 55-kDa binding components in canine renal membrane (19). The multiplicity of apparent specific binding sites for PTH seen by Wright et al. (18) could have been due to proteolysis of the receptors, perhaps associated with internalization of photoligand-receptor complexes at the higher incubation temperatures used, or might have been influenced by the use of a less chemically characterized and biologically active photoprobe. Alternatively, as proposed by Wright et al. (18), the multiple labeled macromolecules might reflect additional components of the receptor which may be a multimeric protein as in the case of the insulin receptor (32). Our results are more similar to those of Nissenson et al. (19), in their studies of PTH receptors in canine renal cortex membranes identified by covalent labeling with a heterobifunctional cross-linking reagent. These investigators emphasized, as we also do in this study, the importance of demonstrating high-affinity of the PTH binding component, if it is to be considered an authentic, physiologically relevant PTH receptor. We have no clear explanation for the detection of apparently specific binding components of 130, 85, and 55 kDa by Nissenson et al. (19). This heterogeneity of apparently specific binding sites may be due to protease activity at the higher incubation temperature these investigators used or perhaps to the well known nonspecificity inherent in chemical cross-linking techniques (33, 34). It is noteworthy that a macromolecule with an apparent molecular weight of 70 kDa, close in size to the principal components recognized by Wright et al. (18) and in earlier studies (15–17), could be identified when protease inhibitors were omitted during isolation of the plasma membranes and, presumably, was derived from the 85-kDa labeled macromolecule (19).

The identity of the other labeled components we identified remains unknown. Since they persisted in the presence of more than 2,500-fold molar excess of NlePTH, did not show saturation kinetics, and were present after cells were preincubated for several days with saturating doses of NlePTH, it seems quite unlikely that they are directly related to PTH receptors in ROS 17/2.8 cells. Furthermore, as described in the accompanying report (28), brief rinsing with acidic PBS (pH 2.5, 4 °C) of the cells incubated with radiodinated NAP-NlePTH, which resulted in selective and nearly complete disappearance of the labeled $M_0 = 80,000$ protein, did not influence labeling of most of these components, including the $M_0 = 70,000, 42,000, 29,000$, and $22,000$ bands, suggesting that these nonspecifically labeled proteins are intracellular or in inaccessible portions of the plasma membrane.

The $M_0 = 80,000$ protein, the only macromolecule that binds to the photoligand with properties characteristic of a physiologically relevant receptor, is likely to represent the entire receptor molecule. Alternatively, it could represent a binding subunit of a dissociated polymeric structure in which only one subunit binds the photoaffinity label, as with the insulin receptor (32). The stability of the $M_0 = 80,000$ protein to strong reducing agents, however, argues against the notion that the PTH receptor consists of more than one structurally different subunit covalently linked at least by sulphydryl bonds.

We believe that the present results, using photoaffinity labeling of specific receptors, will serve to guide future studies of putative PTH receptors in various rat tissues and in other
species. Finding a single, high molecular weight protein with the relevant binding characteristics which, as shown in the accompanying report, is also associated with specific chemical properties relating to complex glycosylated side chains should be useful in future efforts to determine the similarity or differences between macromolecules thought to represent PTH receptors in renal and osseous tissues, other cell lines of interest, and lymphocytes recently reported by us and others to contain high-affinity PTH receptors (36, 37). Furthermore, the recent demonstration by several groups that a polypeptide that is distinct from PTH, but with chemical and biological homology, may be a common cause of the humoral hypercalcemia of malignancy syndrome (38-40) raises the possibility that other receptors specific for this molecule may be similar to, yet distinct from, the PTH receptor.

The general approach of photoaffinity labeling of receptors is also useful in efforts to isolate sufficient quantities of the receptor protein to provide partial sequence information, which, in turn, can be used to construct oligonucleotide probes for receptor cloning by recombinant DNA techniques. It is, of course, imperative in this approach to provide maximum assurance that such labeled moieties show the physicochemical and biological characteristics of physiological PTH receptors and are not unrelated plasma membrane components. Our results permit us to calculate the feasibility of using photoaffinity labeling techniques to isolate sufficient receptor protein for protein microsequencing. Direct quantitation of photoaffinity labeling techniques to isolate sufficient receptor and are not unrelated plasma membrane components.

REFERENCES

Photoaffinity Labeling of PTH Receptor

In this study, we used the photoaffinity labeling technique to investigate the binding properties of the PTH receptor.

Materials and Methods

Photoaffinity labeling was performed using a photolabile PTH

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Materials: [List of materials used in the experiment]

Methods: [Description of the experimental procedure]

Results: [Presentation of the results obtained from the experiment]

Discussion: [Analysis and interpretation of the results]

Conclusion: [Summary of the findings and implications]

Acknowledgments: [Acknowledgment of contributions from other individuals or institutions]

References: [List of references cited in the manuscript]

Supplementary Material: [Additional information or data that supports the findings]

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Results

In vivo biological evaluation of [125I]-labeled MAP-AFP, MAP-AFP was shown to be a highly active agent in various different in vivo and in vitro assays for the detection of the PTH receptor. The biological activity of MAP-AFP was determined by measuring the incorporation of labeled MAP-AFP into RNA and DNA in cultured cells. The results showed that MAP-AFP had a high binding affinity to the PTH receptor, with an IC50 value of 10 nM in vitro.

Figure 1: Biological activity of [125I]-labeled MAP-AFP. A: Competition inhibition by non-labeled MAP-AFP and MAP-AFP. B: Effect of non-labeled MAP-AFP on [125I]-labeled MAP-AFP binding to PTH receptor in vitro. The IC50 values of non-labeled MAP-AFP were 10 nM and 100 nM, respectively. C: Effect of non-labeled MAP-AFP on [125I]-labeled MAP-AFP binding to PTH receptor in vivo. The IC50 values of non-labeled MAP-AFP were 10 nM and 100 nM, respectively.

Figure 2: Northern blot analysis of [125I]-labeled MAP-AFP. A: The radiolabeled protein profile of [125I]-labeled MAP-AFP was detected using SDS-PAGE and autoradiography. B: The radiolabeled protein profile of [125I]-labeled MAP-AFP was detected using SDS-PAGE and autoradiography. C: The radiolabeled protein profile of [125I]-labeled MAP-AFP was detected using SDS-PAGE and autoradiography.

Table 1: Biochemical characterization of [125I]-labeled MAP-AFP. The binding affinity of [125I]-labeled MAP-AFP to PTH receptor was determined by displacement experiments with various concentrations of unlabeled MAP-AFP. The IC50 value of [125I]-labeled MAP-AFP was 10 nM.

<table>
<thead>
<tr>
<th>Condition</th>
<th>IC50 (nM)</th>
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<tbody>
<tr>
<td>Non-labeled MAP-AFP</td>
<td>100</td>
</tr>
<tr>
<td>[125I]-labeled MAP-AFP</td>
<td>10</td>
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*Data are expressed as the mean ± SE of two separate experiments.
Photoaffinity Labeling of PTH Receptor

Figure 1. Time course of $^{35}$S-labeled PTH binding to ROS 17/2.8 cells.

Guinea pig cells were incubated in the dark for 1 hour with radiolabeled PTH (30 pmol) for varying periods of time as described previously. (A) Total binding, (B) specific binding, and (C) nonspecific binding. The data are the means of duplicate determinations and are representative of two similar experiments.

Figure 2. Specificity of $^{35}$S-labeled PTH binding to PTH receptors on ROS 17/2.8 cells. Specific binding of radiolabeled PTH (2000 cpm; 50 pmol) to cells was determined in the absence (basal binding) and in the presence of varying concentrations of unlabeled PTH (200, 300, 400, 500, 1000, 2000 pmol) or DPDPE (100-10,000 pmol) using PTH (1-34) (B) and human ACTH (1-24) (C) (data provided by Dr. Jin Cheng, University of California, San Francisco). The maximal total binding of $^{35}$S-labeled PTH was 1.2% of the added total radioactivity. Nonspecific binding (2% of total binding) was determined and specific binding calculated as the difference in specific binding.

Figure 3. Saturation analysis of PTH receptors in ROS 17/2.8 cells. Guinea pig cells (2 X 10^6 cells/well) were incubated in the dark for 1 hour with incremental doses of radiolabeled PTH (0.01-1000 pmol) and NHP-PTH, both labeled to a specific activity of NHP-PTH 120 CPM/mmol with a mixture of $^{35}$S- and $^{33}$P-PTH, as described previously. Specific and nonspecific binding were determined in duplicate. In all cases, the S/N value was greater than 15 of the same volume. Nonspecific binding was determined, and specific binding was calculated as the difference. Specific binding of radiolabeled PTH (0-1000 pmol) and NHP-PTH (0-1000 pmol) was plotted as a function of increasing ligand dose. Similar saturation analysis of the same data, radiolabeled PTH (0-1000 pmol) and NHP-PTH (0-1000 pmol), was performed using ROS 17/2.8 cells. The data are representative of two similar experiments.

Figure 4. Specific inhibition of PTH binding in ROS 17/2.8 cells. Specific binding of radiolabeled PTH (10,000 cpm; 50 pmol) to cells was determined in the presence of varying concentrations of unlabeled PTH (10, 50, 100, 200, 500, 1000, 2000 pmol) or DPDPE (100-10,000 pmol) using PTH (1-34) (B) and human ACTH (1-24) (C) (data provided by Dr. Jin Cheng, University of California, San Francisco). The maximal total binding of $^{35}$S-labeled PTH was 1.2% of the added total radioactivity. Nonspecific binding (2% of total binding) was determined and specific binding calculated as the difference. Specific binding of radiolabeled PTH (0-1000 pmol) and NHP-PTH (0-1000 pmol) was plotted as a function of increasing ligand dose. Similar saturation analysis of the same data, radiolabeled PTH (0-1000 pmol) and NHP-PTH (0-1000 pmol), was performed using ROS 17/2.8 cells. The data are representative of two similar experiments.