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The Canine Renal Parathyroid Hormone Receptor Is a Glycoprotein: Characterization and Partial Purification†

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ABSTRACT: Covalent labeling of the canine renal parathyroid hormone receptor with [^{125}I]bPTH(1–34) reveals several major binding components that display characteristics consistent with a physiologically relevant adenylate cyclase linked receptor. Through the use of the specific glycosidases neuraminidase and endoglycosidase F and affinity chromatography on lectin–agarose gels, we show here that the receptor is a glycoprotein that contains several complex N-linked carbohydrate chains consisting of terminal sialic acid and penultimate galactose in a β1,4 linkage to *N*-acetyl-D-glucosamine. No high mannose chains or O-linked glycans appear to be present. The peptide molecular weight of the deglycosylated labeled receptor is 62 000 [or 58 000 if the mass of bPTH(1–34) is excluded]. The binding of [^{125}I]bPTH(1–34) to the receptor is inhibited in a dose-dependent fashion by wheat-germ agglutinin, but not by either succinylated wheat-germ agglutinin or *Ricinus communis* lectin, suggesting that terminal sialic acid may be involved in agonist binding. A combination of lectin affinity chromatography and immunoaffinity chromatography affords a 200-fold purification of the covalently labeled receptor.

It is generally agreed that most of the effects of parathyroid hormone (PTH)[1] on its major targets (kidney and bone) are mediated, at least in part, by an intrinsic membrane-bound receptor which is catalytically linked to adenylate cyclase through a stimulatory guanine nucleotide regulatory protein (G_s) (Goltzman et al., 1978; Nissenson, 1982; Teitelbaum et al., 1982; Habener et al., 1984). Whereas the biological properties of this receptor have been characterized extensively, little information on its structure has been forthcoming since its initial identification by photoaffinity radiolabeling techniques (Coltrera et al., 1981; Draper et al., 1982). It has been necessary to covalently label the receptor in order to detect its presence after detergent solubilization, because disruption of the receptor–G_s complex shifts the receptor to a very low affinity state for agonist binding (Goltzman et al., 1978; Teitelbaum et al., 1982), and no ligands (either PTH agonists or antagonists) of sufficient affinity to be useful for detection of the low-affinity state of the PTH receptor are currently

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[1] Abbreviations: PTH, parathyroid hormone; b, bovine; h, human; VIP, vasoactive intestinal peptide; HSAB, *N*-succinimidyl 4-azidobenzoate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid trisodium salt; GTP, guanosine 5′-triphosphate; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; WGA, wheat-germ agglutinin; S-WGA, succinylated wheat-germ agglutinin; RCA_I, *Ricinus communis* agglutinin I; Con A, concanavalin A; PNA, peanut agglutinin; UEA-F, *Ulex europaeus* agglutinin I; GlcNAc, *N*-acetyl-D-glucosamine; Gal, D-(+)-galactose; Man, methyl α-D-mannopyranoside; GTP, guanosine 5′-triphosphate; Gpp(NH)p, guanylyl imidodiphosphate; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); App(NH)p, adenylyl imidodiphosphate; G_s, stimulatory guanine nucleotide regulatory component of adenylate cyclase; Iodo-Gen, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Triton, Triton X-100; kDa, kilodaltons; DMSO, dimethyl sulfoxide; Ig, immunoglobulin.

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available. Furthermore, there have been no reports to date of even initial stages of purification of this low-abundance membrane protein. This is due in large part to the lack of suitable reagents (i.e., anti-receptor antibodies) as well as the inability to follow binding activity of the solubilized receptor throughout various purification steps.

Recent work in our laboratory, using the heterobifunctional cross-linking reagent HSAB, has identified a predominant M_r[2] 85 000 PTH binding protein in canine renal plasma membranes isolated in the presence of protease inhibitors, which decreases in apparent mass to M_r 70 000 in the absence of protease inhibitors (Nissenson et al., 1987). Minor bands also are seen on SDS–PAGE with M_rs of 170 000, 135 000, 55 000, and <14 400. Labeling of all bands can be inhibited by the inclusion in the binding reaction of as little as 1 nM unlabeled PTH, which correlates well with the K_d of the receptor in binding competition assays and with the K_m for adenylate cyclase activity (Teitelbaum et al., 1982). Labeling also is fully inhibited by coincubation with 100 μM Gpp(NH)p or GDPβS but not with 100 μM GMP or App(NH)p, as would be expected of a receptor that is coupled to G_s. The predominant M_r 85 000 band appears to represent the binding component of the physiologically relevant PTH receptor.

Many transmembrane hormone receptors recently have been shown to be glycosylated, including the adenylate cyclase linked receptors for glucagon (Iyengar & Herberg, 1984; Herberg et al., 1984; Iyengar & Herberg, 1985; Iwanij & Hur, 1985), VIP (Nguyen et al., 1986), and β-adrenergic agents (Stiles et al., 1984; Cervantes-Olivier et al., 1985). The presence of carbohydrate on receptors has allowed the use of lectin affinity chromatography for their purification (Hedo, 1984) and in the case of the cyclase-linked glucagon receptor has provided a method for assaying the free, solubilized receptor unassociated with G_s (Herberg et al., 1984). If the PTH receptor likewise is glycosylated, these or similar techniques could significantly enhance its further characterization and purification. In particular, lectin affinity chromatography might provide a means of studying the solubilized PTH receptor disassociated from G_s.

The results of the present study indicate that the canine renal PTH receptor is a glycoprotein containing several complex N-linked carbohydrate chains, which account for approximately 23 kDa of the apparent molecular mass of the receptor on SDS–PAGE. Terminal sialic acid residues are present on at least one of the carbohydrate chains and may be in close proximity to the site of hormone binding. Finally, we report the use of WGA affinity chromatography in the partial purification of the covalently labeled PTH receptor.

Materials and Methods

Materials. All agarose-bound lectins, as well as free WGA, S-WGA, RCA_I, Con A, PNA, and UEA-F, were from Vector Laboratories (Burlingame, CA). Neuraminidase from *Clostridium perfringens* (EC 3.2.1.18) (160 units/mg), PMSF, pepstatin, leupeptin, NEM, *N*-acetyl-D-glucosamine, D-(+)-galactose, L-fucose, methyl α-D-mannopyranoside, and *N*,*N*′-diacetylchitobiose were from Sigma Chemical Co. (St. Louis, MO). HSAB, Iodo-Gen, and Triton X-100 were from Pierce Chemical Corp. (Rockford, IL), aprotinin was from Mobay Chemical Corp. (New York, NY), heat-inactivated *Staphylococcus aureus* (IgGsorb) was from Enzyme Center, Inc. (Waltham, MA), and bovine PTH(1–34) (5000 units/mg) was from Bachem (Torrance, CA). Centricon 10 microconcentrators were from Amicon (Danvers, MA), and Spectropor 2 dialysis tubing was from Spectrum Medical Industries (Los Angeles, CA). NP-40 was from Particle Data Labs, Ltd. (Elmhurst, IL). Acrylamide gradient gels (5–15% and 5–20%) for SDS–PAGE were purchased from Isolab (Barberton, OH). $Na^{125}I$ was from Amersham, Inc. (Arlington Heights, IL), and $Na^{131}I$ was from Cintichem, Inc. (Tuxedo, NY). Purified endoglycosidase F (Endo F) was the very generous gift of Drs. John H. Elder and Stephen Alexander, Scripps Clinic and Research Foundation; its preparation has been previously described (Elder & Alexander, 1982). Extensive use of this enzyme preparation with hundreds of proteins has failed to reveal any proteolytic activity in the presence of EDTA (J. Elder, personal communication). All other chemicals were from Sigma.

Isolation of Canine Renal Plasma Membranes. Canine renal cortical plasma membranes were isolated by a published modification (Nissenson & Arnaud, 1979) of the technique of Fitzpatrick et al. (1969). Such preparations are enriched 7–10-fold (over crude homogenates) in ouabain-sensitive Na^+,K^+-ATPase. Kidneys were processed in buffers containing either leupeptin (45 μg/mL) and EDTA (1.0 mM) or a mixture of protease inhibitors consisting of PMSF (10 μg/mL), leupeptin (5 μg/mL), pepstatin (5 μg/mL), aprotinin (10 units/mL), NEM (1.0 mM), and EDTA (1.0 mM). Membranes prepared with both combinations manifested essentially identical autoradiographic patterns on SDS–PAGE following covalent labeling. Membranes were stored at −80 °C and were stable with respect to [^{125}I]bPTH(1–34) binding activity for at least 6 months.

Iodination of Canine Renal Plasma Membranes. Canine renal plasma membranes were iodinated with $Na^{125}I$ and Iodo-Gen as previously described (Markwell & Fox, 1978). Plasma membranes (500 μg) in 100 μL of 50 mM Tris-HCl, 50 mM HEPES (pH 7.5), 2 mM $MgCl_2$, and 7.5 μL (5 mCi) of $Na^{125}I$ were added to a plastic microfuge tube onto which 100 μg of Iodo-Gen had previously been evaporated. Iodination was allowed to proceed for 18 min at room temperature, at which point the reaction mixture was diluted to 1.5 mL with ice-cold 50 mM Tris-HCl, 50 mM HEPES (pH 7.5), 0.1% BSA, and 2 mM $MgCl_2$. This solution was immediately transferred to a glass centrifuge tube containing 6 mL of ice-cold 50 mM Tris-HCl, 50 mM HEPES (pH 7.5), 0.25 M NaI, and 0.1% BSA, and the membranes were washed by centrifiguation and resuspension three times prior to storage at −80 °C.

[^{125}I]bPTH(1–34) and [^{131}I]bPTH(1–34) Receptor Cross-Linking. Biologically active, electrolytically labeled [^{125}I]bPTH(1–34) and [^{131}I]bPTH(1–34) were prepared and purified by high-performance liquid chromatography as previously described (Nissenson et al., 1986). Approximately 1.0 μCi/mL of [^{125}I]bPTH(1–34) was incubated for 2 h at 30 °C with canine renal membranes (250 μg/mL) in a solution consisting of 50 mM Tris-HCl, 50 mM HEPES (pH 7.5), 2.0 mM $MgCl_2$, and 0.1% BSA. Incubates were microcentrifuged at 4 °C, and the pellets were resuspended in ice-cold 50 mM sodium phosphate (pH 7.6) containing 0.1% BSA. After centrifugation, this wash step was repeated. Membrane pellets were washed an additional time with 50 mM sodium phosphate (pH 7.6, no BSA) and were resuspended in this buffer. The heterobifunctional cross-linking reagent HSAB in DMSO was

[2] Molecular weight estimates for receptor components correspond to the actual position of labeled bands on SDS–PAGE without correction for the contribution of [^{125}I]bPTH(1–34) and HSAB, unless otherwise indicated. Assuming a 1:1:1 stoichiometry for [^{125}I]bPTH(1–34):HSAB:binding protein, the estimates of receptor M_r would be ~4500 less than those presented.

added to a final concentration of 0.5 mM, and incubation was carried out for 10 min on ice in the dark. The reaction was terminated by the addition of 2 M Tris-HCl (pH 7.5). Samples were transferred to 24-well cluster plates and subjected to photolysis for 20 min using a Blak-Ray ultraviolet lamp (emission maximum 365 nm) at a distance of 8 cm. The membranes then were washed once with 50 mM sodium phosphate (pH 7.6). Cross-linking with [^{131}I]bPTH(1–34) was carried out in an identical fashion, except that approximately 50 μCi of ^{125}I-labeled membranes were added either following photolysis or prior to binding with [^{131}I]bPTH(1–34). Identical results were obtained with the two procedures. Membrane protein concentrations were determined by the method of Lowry et al. (1951).

Solubilization. After cross-linking, membrane pellets were resuspended at a detergent:protein ratio of 10:1 w/w in solubilization buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0 mM EDTA, and 1.0% Triton X-100. When the sample was to be chromatographed on Con A, PNA, or UEA-F, EDTA was withheld from the solubilization buffer and the appropriate divalent cations were added at a final concentration of 1.0 mM, as these lectins require, respectively, Ca^{2+} and Mn^{2+}, Ca^{2+} and Mg^{2+}, or Ca^{2+} alone in order to maintain binding activity. The resuspended cross-linked pellets were agitated on an orbital shaker for 1.0 h at room temperature, after which the nonsoluble material was separated by centrifugation at 200000*g* for 30 min at 4 °C. When this technique was used, greater than 85% of the cross-linked radioactivity was routinely solubilized.

Lectin Affinity Chromatography. The agarose-bound lectins used in this study and their carbohydrate specificities are as follows: WGA (terminal sialic acid, terminal and internal *N*-acetyl-D-glucosamine); S-WGA (terminal and internal *N*-acetyl-D-glucosamine); RCA_I (terminal and penultimate D-galactose linked β1,4 to *N*-acetyl-D-glucosamine); Con A (terminal and internal α-D-mannose); PNA (terminal D-galactose linked β1,3 to *N*-acetylgalactosamine; and UEA-F (terminal α-L-fucose. Lectin columns with bed volumes of 1.0 mL were regenerated by washing with 50 mL of buffer A containing 0.01% SDS. For WGA, S-WGA, and RCA_I buffer A consisted of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0 mM EDTA, and 0.1% Triton X-100. For the remaining lectins the EDTA was withheld, and the following divalent cations (1 mM) were added: Ca^{2+} and Mn^{2+} (Con A), Ca^{2+} and Mg^{2+} (PNA), or Ca^{2+} alone (UEA-F). After the SDS wash, the columns were washed with 25 mL of buffer A containing 300 mM of the appropriate haptenic sugar (buffer B), followed by at least 200 mL of buffer A alone. All steps were carried out at 4 °C. Solubilized preparations of covalently labeled membranes were applied to the columns and recycled twice. The material that failed to adsorb to the column constituted the "run-through". The columns were then washed with 25–50 mL of the appropriate buffer A in order to remove the remainder of the nonspecifically bound material. The final 1.0 mL of wash never contained greater than background radioactivity.

Specifically bound radioactivity was eluted by 1.0-mL washes of buffer B at 0.1 mL/min (this fraction constitutes the "sugar eluate"), with approximately 90% of the bound counts eluting in the first 1.0-mL wash. The specificity of lectin binding was assessed by the addition of the specific haptenic sugar to the solubilized covalently labeled receptor prior to chromatography. When lectin column fractions were assessed by SDS–PAGE and autoradiography, equivalent aliquots of the run-through and sugar-eluate fractions were utilized so that direct comparisons could be made.

Neuraminidase Treatment. Cross-linked membranes were washed once with 100 mM sodium acetate (pH 5.1) at 25 °C, followed by centrifugation at 18000*g* for 10 min. Membranes were then resuspended in the same buffer containing 0.1 mM PMSF, 3 μg/mL pepstatin, and 5 mM EDTA. Neuraminidase was added at a concentration of 1.0 unit/mL to all except the control sample, and incubation was performed for 14 h at 37 °C. An aliquot of control and neuraminidase-treated membranes was removed, centrifuged at 18000*g*, washed once with 20 mM Tris-HCl (pH 7.4), and solubilized in electrophoresis buffer for subsequent SDS–PAGE or in solubilization buffer for lectin chromatography.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Samples to undergo SDS–PAGE were dialyzed against 20 mM Tris-HCl (pH 7.4) and 0.1% SDS at 4 °C overnight, utilizing Spectropor 2 dialysis tubing with a molecular weight cutoff of 12 000–14 000. Samples were concentrated by Centricon 10 centrifugation, adjusted to a final concentration of 76.3 mM Tris-HCl (pH 6.8), 9.8% glycerol, 1.06% SDS, and 0.46% dithiothreitol, and incubated at 37 °C for 10 min. Boiling of samples was avoided since this led to apparent aggregation, with retention of the samples in the 3% stacking gel during electrophoresis. Samples then were subjected to SDS–PAGE according to the method of Laemmli (1970), using gradient slab gels containing either 5–15% or 5–20% acrylamide in the separating gel. Following electrophoresis the gels were fixed and stained with Coomassie blue G-250, destained, and dried with a Bio-Rad gel dryer prior to autoradiography at −80 °C. The dried gels were exposed to Kodak X-Omat film in cassettes equipped with a single intensifying screen (Cronex Lightening-Plus). Molecular weights were determined from plots of log molecular weight versus log R_F (relative migration, measured at the center of the band). The molecular weight markers used (Bio-Rad) were myosin (M_r 200 000), β-galactosidase (M_r 116 000), phosphorylase *b* (M_r 92 500), bovine serum albumin (M_r 66 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and lysozyme (M_r 14 400).

Endoglycosidase F Treatment. Covalent labeled receptor that had been purified by WGA chromatography was dialyzed overnight and subjected to SDS–PAGE on a 5–20% gradient gel as described above. Upon completion of the electrophoresis the sample lane was sliced on a Bio-Rad Model 195 electric gel slicer, and the slices were counted on a Packard Multi-Prias 4 γ counter. The slices containing the 85-kDa receptor component were pooled and transferred to the sample cup of an Isco Model 1750 sample concentrator, overlaid with 10 mM NH_4HCO_3 (pH 8.6) and 0.02% SDS, and electroeluted at 1 W for 12 h at 4 °C. An identical procedure was carried out for the 55-kDa component. Recovery of components from the gel slices by this technique averaged >70%. The electroeluted material was dialyzed overnight against 100 mM sodium phosphate (pH 6.1), 50 mM EDTA, and 0.1% SDS at 4 °C and then concentrated by Centricon 10 centrifugation at room temperature for 60 min. Endo F treatment was then performed according to the protocol of Elder and Alexander (1982). The isolated 85- and 55-kDa receptor species were each divided into two equal aliquots, and the volume was adjusted to 90 μL with 100 mM sodium phosphate (pH 6.1), 50 mM EDTA, 1.0% β-mercaptoethanol, and 0.1% SDS and incubated at 30 °C for 3 min. The samples were then made 1.0% with respect to NP-40, and 30 units/mL of Endo F or buffer alone was added. The samples were incubated at 37

°C for 18 h, after which they were prepared for SDS–PAGE as described above.

Receptor Binding Assays. PTH binding in canine renal plasma membranes was determined in triplicate as described previously (Nissenson et al., 1985), except that membranes were incubated for 60 min at 30 °C in the absence or presence of increasing concentrations of free lectins prior to the 2-h incubation with [^{125}I]bPTH(1–34). At the highest lectin concentration, controls were included that contained a 100-fold excess of each lectin's specific saccharide hapten; in the case of WGA and S-WGA this was *N*,*N*′-diacetylchitobiose (*N*-acetyl-D-glucosaminyl-β1,4-*N*-acetyl-D-glucosamine). Additionally, bound and free hormone was separated by microcentrifugation rather than by filtration. Blank binding was negligible, and nonspecific binding, which was assessed in the presence of 1 μM bPTH(1–34), was routinely <0.1% of the total binding and was not significantly altered by the presence of lectins. Specific binding represents total minus nonspecific binding.

Initial Purification of the [^{131}I]bPTH(1–34) Cross-Linked Receptor. Canine renal plasma membranes were labeled with ^{125}I, mixed with unlabeled membranes, and then cross-linked to [^{131}I]bPTH(1–34) as described above. Purification was then performed by a combination of lectin chromatographic and immunoaffinity techniques as outlined in Table V. Lectin chromatography on WGA, S-WGA, and RCA$_1$ was performed as described above; details of the immunoaffinity procedures are given in succeeding sections. The degree of purification achieved was assessed by an increase in the ratio of ^{131}I (PTH receptor) to ^{125}I (membrane protein) radioactivity. All values for ^{125}I radioactivity are corrected for the ^{131}I crossover.

Immunoprecipitation of the Solubilized, Covalently Labeled PTH Receptor. Canine renal plasma membranes were cross-linked to [^{125}I]bPTH(1–34), solubilized, and twice-purified over a WGA affinity column as described above. Following overnight dialysis at 4 °C against 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Triton X-100 ("buffer"), 100 μL of this solubilized material was incubated with 10 μL of 1:10 normal goat serum or goat anti-hPTH(1–34) serum (anti-PTH serum) at 4 °C for 27 h and subsequently precipitated for 24 h at 4 °C with 100 μg of rabbit anti-goat IgG. A second precipitation was performed with excess heat-inactivated *S. aureus* for 4 h at 23 °C. Insoluble material was pelleted by microcentrifugation at 13800*g*, and the pellets were washed 2 times with buffer. The antiserum used was developed by one of us (C.D.A.) in goats against synthetic hPTH(1–34) and has specificity for the amino terminus of hPTH. It exhibits high affinity for hPTH(1–34) with cross-reactivity for bPTH(1–34).

Immunoaffinity Chromatography. [^{131}I]bPTH(1–34) ^{125}I-labeled (^{131}I/^{125}I-labeled) membranes were prepared as described above, but solubilization was carried out with 1.0% Triton at an increased detergent:protein ratio of 100:1. This material was purified by a single passage over WGA–agarose. The first milliliter of *N*-acetyl-D-glucosamine eluate was diluted to 4 mL with buffer A and applied to a column containing 300 μL of anti-PTH IgG–Sepharose CL-4B that had been equilibrated with buffer A. The IgG used was an ammonium sulfate fraction of the anti-hPTH antiserum used for the immunoprecipitation experiments. Incubation was carried out by constant rotation at 4 °C for 17 h. The 4-mL run-through was collected, and the column was washed with buffer A until both the eluting ^{131}I and ^{125}I radioactivities had returned to base line. Specific elution was then performed with 2.0 mL of buffer A containing 1.0 μg/mL bPTH(1–34), which was allowed to interact with the column by rotation for 4 h at 23 °C. This fraction was collected, and the column subsequently was eluted with two 1.0-mL aliquots of the PTH-containing buffer.

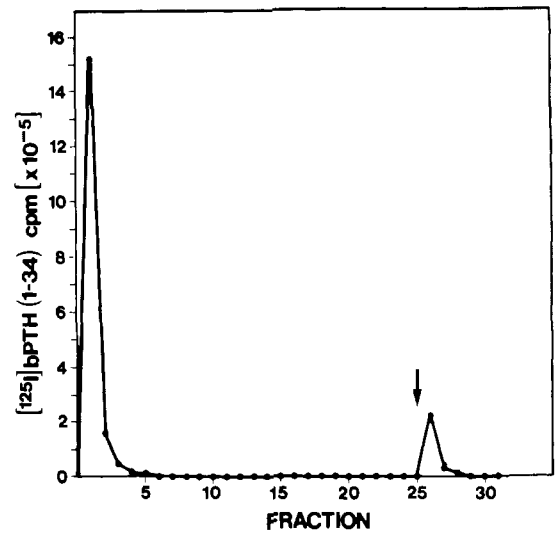

FIGURE 1: Elution profile of solubilized [^{125}I]bPTH(1–34)-labeled canine renal membranes on WGA–agarose. [^{125}I]bPTH(1–34)-labeled canine renal membranes were solubilized in 1.0% Triton X-100, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1.0 mM EDTA and chromatographed on a WGA–lectin column; 2 × 10^6 cpm were applied to the column, and 1.0-mL fractions were collected. Fraction 1 represents the run-through. The column was washed with 0.1% Triton X-100, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1.0 mM EDTA (buffer A) until the radioactivity eluting returned to base line, at which point the column was eluted with buffer A containing 300 mM GlcNAc (arrow). Run-through = nonadsorbed material.

Replication of Experiments and Statistical Analysis. The experiments involving control and post-neuraminidase binding to lectin affinity columns, the effect of free lectins on hormone binding, and the effect of treatment with Endo F and the double-labeling experiments to assess degree of purification were performed at least twice. All other experiments were preformed at least 3 times with different preparations of membranes. Qualitatively similar results were observed, and hence representative experiments are shown. Results were analyzed by Student's *t*-test where applicable; values given represent the mean ± the SEM.

RESULTS

Lectin Chromatography. Initial experiments were carried out to determine whether the renal PTH receptor might display affinity for various carbohydrate-binding lectins. In these studies, the membrane-associated PTH receptor was covalently labeled with [^{125}I]bPTH(1–34), solubilized with 1.0% Triton X-100, and chromatographed on a variety of agarose–lectin affinity columns. A representative chromatographic profile on WGA–agarose is shown in Figure 1. More than 85% of the applied ^{125}I radioactivity appeared in the column run-through or in the initial washes and, hence, failed to adsorb to the WGA–agarose. When this unadsorbed fraction was analyzed by SDS–PAGE (Figure 2A), essentially all of the ^{125}I radioactivity appeared at the dye front, as would be expected of [^{125}I]bPTH(1–34). We interpret this result to indicate that 85% of the ^{125}I radioactivity in Triton-solubilized membranes was unbound [^{125}I]bPTH(1–34) that had dissociated from the receptor during the cross-linking or subsequent solubilization procedures. About 15% of the applied radioactivity adsorbed to the WGA–agarose column, and most (>75%) of the adsorbed ^{125}I radioactivity was eluted with the specific sugar GlcNAc. SDS–PAGE of the GlcNAc eluate

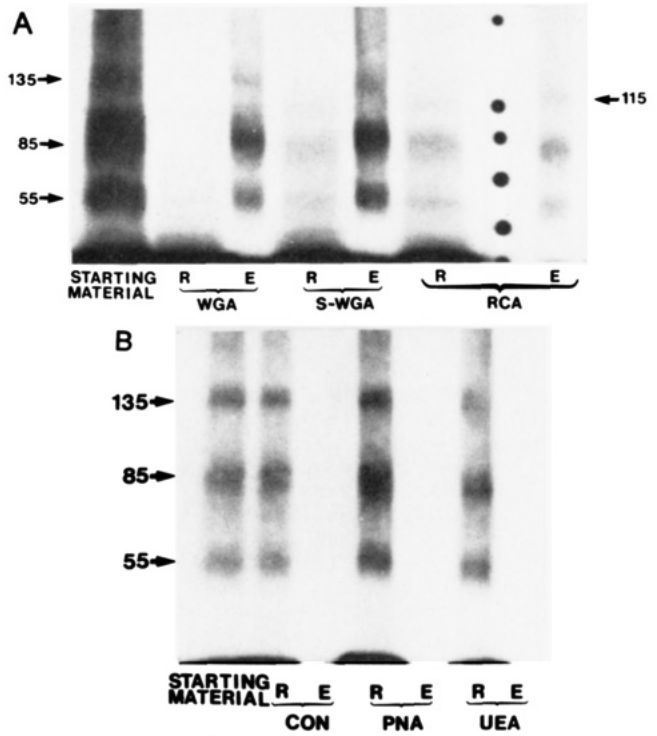

FIGURE 2: Lectin affinity chromatography of CRPMs covalently labeled with [^{125}I]bPTH(1–34). Following solubilizing of the covalently labeled receptor, chromatography was performed on a panel of agarose-bound lectins as described in the legend for Figure 1. Equal aliquots of the nonretained material (run-through) and the specifically eluted material (sugar elutate) were run on SDS–PAGE, and the gel was subjected to autoradiography. R = run-through; E = sugar eluate. (A) Results with WGA, S-WGA, and RCA (RCA_I). The dots are molecular weight standards that were overlaid with ^{36}Cl. (B) Results with CON (Con A), PNA, and UEA (UEA-F). Molecular weights shown are ×1000.

Table I: Affinity of the PTH Receptor for Various Solid-Phase Lectins[a]

lectin	n	% of applied radioactivity specifically eluted
WGA	13	11.7 ± 1.0
S-WGA	4	9.7 ± 2.5
RCA_I	4	8.0 ± 2.2
Con A	3	0.9 ± 0.1
PNA	3	0.4 ± 0.4
UEA	3	0.4 ± 0.2

[a] Canine renal membranes were covalently labeled with [^{125}I]bPTH-(1–34), solubilized, and chromatographed over various lectin affinity columns as outlined under Materials and Methods. The radioactivity that was eluted only with each lectin's specific sugar is given as a percentage of the applied radioactivity (10^5 to 5×10^5 cpm). The results are the mean ± SEM of n experiments.

revealed the presence of the ^{125}I-labeled 85-, 135-, and 55-kDa membrane components that were present in the starting material[3] (Figure 2A). Our previous studies have provided evidence that these labeled components are constituents of the adenylate cyclase coupled renal PTH receptor (Nissenson et al., 1987).

[3] The pattern routinely seen following Triton X-100 solubilization is somewhat different from that found when cross-linked membranes are immediately analyzed by SDS–PAGE. In the latter condition there is (in membranes prepared with protease inhibitors) a clearly dominant 85-kDa band, a less prominent band of <14 400 kDa, and very minor bands at 170, 135, 55, and 40 kDa. In contrast, when covalently labeled membranes are solubilized in Triton X-100, there are only labeled bands of 85, <14.4, 55, and 135 kDa, with a relative enhancement of the latter two (although the 85-kDa band remains predominant). We interpret these latter findings as evidence for the presence of proteolysis during the 24 h at 4 °C required for lectin chromatography and dialysis. As a 5–15% acrylamide gel was used in this experiment, the <14.4-kDa band ran at the dye front.

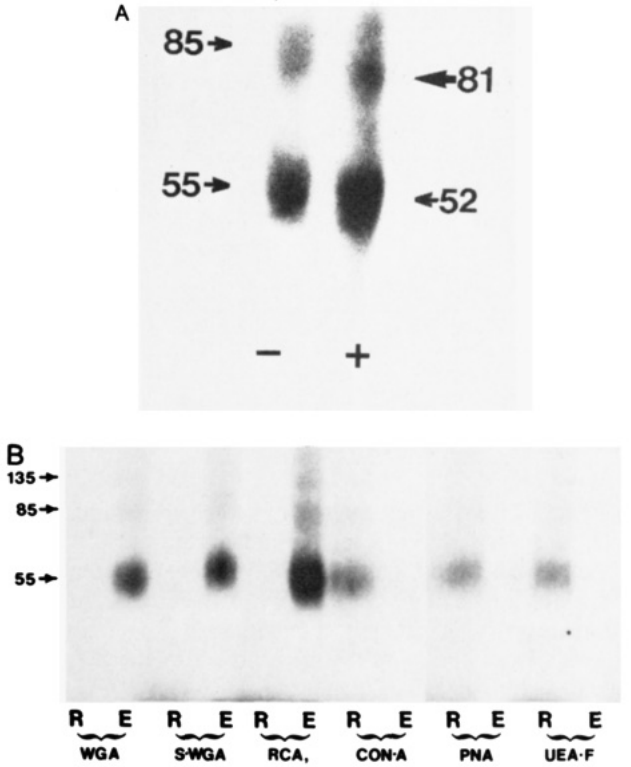

FIGURE 3: Lectin affinity chromatography of canine renal PTH receptor following neuraminidase treatment. Autoradiogram of SDS–PAGE of the indicated fractions. Canine renal membranes were covalently labeled with [^{125}I]bPTH(1–34) as described under Materials and Methods. The labeled membranes were then washed and suspended in 100 mM sodium acetate (pH 5.1) containing 0.1 mM PMSF, 3 μg/mL pepstatin, and 5 mM EDTA. Neuraminidase at 1.0 unit/mL was added to one sample, the control sample receiving buffer alone (see Materials and Methods), and incubation was performed at 37 °C for 14 h. Following sedimentation at 14000g the membranes were directly analyzed by SDS–PAGE (A) or were solubilized and chromatographed on the indicated lectin columns (B). The eluted fractions of interest were desalted by dialysis against 20 mM Tris-HCl (pH 7.4) and 0.1% SDS and prepared for SDS–PAGE as described under Materials and Methods. The total radioactivity eluting in each fraction was applied to the gel. The relative molecular weight shown is ×1000. This experiment is representative of three similar experiments.

The affinity of solubilized, ^{125}I-labeled PTH receptors for six solid-phase lectins is depicted in Table I. The PTH receptor bound specifically to S-WGA and RCA_I, as well as to WGA, whereas little or no specific binding to Con A, PNA, or UEA-F was observed. SDS–PAGE revealed that the macromolecular labeled membrane components adsorbed to WGA, S-WGA, and RCA_I (Figure 2A), whereas there was no significant adsorption of any labeled components to Con A, PNA, or UEA-F (Figure 2B). The appearance of macromolecular components in the S-WGA and RCA_I run-through indicates that the adsorption of receptor components was not quantitative for these two lectins.

Retained labeled bands on WGA, S-WGA, and RCA_I could be eluted only by each lectin's specific haptenic sugar, and retention was never observed when the specific sugar was included in the sample before chromatography.

Exoglycosidase Treatment. In order to assess the presence of terminal sialic acid residues, canine renal plasma membranes were covalently labeled with [^{125}I]bPTH(1–34) and then were treated with neuraminidase as outlined under Materials and

Table II: Effect of Neuraminidase Treatment on the Affinity of the PTH Receptor for Various Solid-Phase Lectins[a]

expt	lectin	^{125}I radioactivity specifically eluted (cpm) −	+
1	WGA	2730	1230
	S-WGA	2520	1660
	RCA_I	2060	3570
2	WGA	4020	2030
	S-WGA	3250	2270
	RCA_I	3140	4490

[a] Aliquots of solubilized, covalently labeled PTH receptor were treated in the absence (−) or presence (+) of neuraminidase, as described under Materials and Methods. Samples then were chromatographed on lectin affinity columns, and the specifically eluted ^{125}I radioactivity was collected and counted. About 25 000 and 40 000 cpm were applied to each lectin column in experiments 1 and 2, respectively.

Methods. This enzyme specifically hydrolyzes terminal sialic acid residues. Following neuraminidase treatment, both the 85- and 55-kDa bands showed small but reproducible increases in electrophoretic mobility (Figure 3A). The results of lectin chromatography following neuraminidase treatment are shown in Table II. Treatment with neuraminidase decreased the binding of the solubilized receptor to WGA and S-WGA while increasing the binding to RCA_I; the negligible binding to Con A, PNA, and UEA-F was not altered (data not shown). These results are supported by the findings in Figure 3B, which depict a representative autoradiogram of lectin column run-through and sugar eluate fractions from covalently labeled membranes that have undergone neuraminidase treatment.[4] Treatment under the same conditions but without neuraminidase did not alter the degree of binding to any of the six lectin columns. Whereas prior to neuraminidase treatment the RCA_I run-through lane contained some labeled receptor bands (Figure 2A), indicating subquantitative adsorption, following desialation the RCA_I run-through lane is devoid of labeled bands and there is a relative enhancement of the bands in the sugar eluate lane (Figure 3B). This quantitative adsorption of covalently labeled PTH receptor to RCA_I following desialation is concordant with the increased recovery of ^{125}I radioactivity from this lectin seen in Table II.

Endoglycosidase Treatment. In order to confirm the presence of N-linked carbohydrate, as well as to provide more accurate information regarding the peptide mass of the receptor, the 85- and 55-kDa receptor species were electroeluted after SDS–PAGE and were individually treated with endoglycosidase F, as outlined under Materials and Methods. As shown in Figure 4 treatment for 18 h resulted in conversion of the 85-kDa receptor to a form migrating at about 62 kDa; a minor 75-kDa intermediate form may be seen just above the 62-kDa band. Treatment of the 52-kDa band with Endo F produced forms of 38 and 30 kDa. The canine renal PTH receptor thus appears to contain one or more N-linked glycans, which account for approximately 23 kDa of its apparent mass in the fully glycosylated state.

Effect of Lectins on PTH Receptor Binding. To determine if the lectins possessing affinity for the PTH receptor might alter receptor function, canine renal plasma membranes were

[4] We noted that exposure of the covalently labeled receptor to acid conditions (as for neuraminidase treatment) led to a decrease in the intensity of the 135- and 85-kDa bands, with an apparently commensurate increase in the intensity of the 55-kDa band. This pattern was also seen in the control sample, which underwent incubation at pH 5.1 in the absence of neuraminidase (Figure 3A).

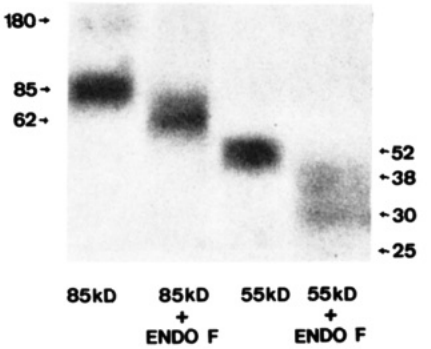

FIGURE 4: Effect of Endo F treatment of the canine renal PTH receptor. Renal membranes covalently labeled with [^{125}I]bPTH(1–34) were solubilized and chromatographed on a WGA affinity column. The GlcNAc eluate was electrophoresed by SDS–PAGE, and the major labeled components (85 and 55 kDa) were isolated and individually electroeluted out of the gel and treated with and without Endo F (30 units/ml) as described under Materials and Methods. Following enzymatic treatment the samples were adjusted to 1.0 mL with 20 mM Tris-HCl (pH 7.4) and 0.1% SDS, dialyzed overnight against the same buffer at 4 °C, concentrated, and analyzed by SDS–PAGE. A photograph of the autoradiogram is shown. The relative molecular weights indicated are ×1000.

Table III: Specific [^{125}I]bPTH(1–34) Binding to Canine Renal Plasma Membranes in the Presence of Various Lectins[a]

lectin	concn (μg/mL)	[^{125}I]bPTH(1–34) specific binding (%)
control	0	17.5 ± 0.4
WGA	25	5.9 ± 0.2[b]
	100	2.3 ± 0.1[b]
	500	1.0 ± 0.5[b]
	500 + diacetylchitobiose	14.9 ± 0.7[b]
S-WGA	25	17.8 ± 1.0
	100	15.3 ± 1.2
	500	19.0 ± 0.6
	500 + diacetylchitobiose	15.1 ± 1.0
RCA_I	25	17.6 ± 0.3
	100	17.0 ± 0.2
	500	12.6 ± 0.6[b]
	500 + D-galactose	10.8 ± 1.2[c]
control	0	13.7 ± 2.3
Con A	25	12.8 ± 1.1
	100	11.7 ± 1.6
	500	11.5 ± 0.7
	500 + methyl α-D-mannopyranoside	13.4 ± 0.8
PNA	25	14.1 ± 2.1
	100	13.1 ± 1.5
	500	12.7 ± 1.2
	500 + D-galactose	13.7 ± 1.5
UEA-F	25	15.7 ± 1.5
	100	18.2 ± 1.5
	500	12.7 ± 1.3
	500 + α-fucose	13.3 ± 1.3

[a] Experimental conditions and [^{125}I]bPTH(1–34) binding were determined as described under Materials and Methods. Results are representative of two experiments run in triplicate. [b] $p < 0.01$, two-tailed. [c] $p < 0.05$, two-tailed.

incubated with various concentrations of non-agarose bound WGA, S-WGA, RCA_I, Con A, PNA, and UEA-F prior to determination of specific [^{125}I]bPTH(1–34) binding. As shown in Table III, specific binding was significantly inhibited by WGA in a dose-related manner, with 66% inhibition by the lowest concentration of this lectin (25 μg/mL). In contrast, specific binding was otherwise inhibited by only the highest concentration of RCA_I (500 μg/mL) and not at all by S-WGA or the other lectins tested. In the case of WGA, but not RCA_I, the inhibition was reversible by coincubation with the specific lectin's sugar but not by sucrose (present in the binding buffer for all samples).

Table IV: Immunoprecipitation of the Covalent [^{125}I]bPTH–Receptor Complex by Goat Anti-PTH(1–34) Serum[a]

	^{125}I radioactivity precipitated (cpm)	
	−	+
anti-PTH(1–34) serum	2070 ± 170	110 ± 70
control serum	390 ± 150	310 ± 70

[a] The solubilized, covalent [^{125}I]bPTH(1–34)–receptor complex was purified by WGA affinity chromatography. Immunoprecipitation of the complex (10000 cpm) was carried out as described under Materials and Methods using goat anti-PTH(1–34) serum or normal goat serum in the presence (+) or absence (−) of a saturating concentration (25 μg/mL) of bPTH(1–34). The values are the mean ± SEM of three experiments.

Table V: Partial Purification of the PTH Receptor by Lectin- and Anti-PTH Affinity Chromatography

procedure	ratio of [^{131}I]PTH receptor: [^{125}I]membrane proteins (×10^3)	purification (x-fold)
expt 1[a]		
crude solubilized receptor	1.87[c]	1
WGA chromatography	19.6	11
S-WGA chromatography	16.7	8.9
RCA_I chromatography	14.9	8.0
expt 2[b]		
crude solubilized receptor	3.38[c]	1
WGA chromatography	86.9	26
anti-PTH affinity chromatography	679	200

[a] Solubilization was carried out at a Triton X-100:protein ratio of 10:1. [b] Solubilization was carried out at a Triton X-100:protein ratio of 100:1. [c] Corrected for efficiency of covalent bond formation, which averaged 11% of membrane-bound [^{125}I]bPTH(1–34).

Purification of the PTH Receptor. We tested the utility of lectin affinity chromatography as a first step in the purification of the covalently labeled PTH receptor. In order to accurately assess purification, an ^{131}I/^{125}I-labeled membrane preparation was employed as outlined under Materials and Methods. This allowed us to follow changes in the ratio of ^{131}I (PTH receptor) radioactivity to ^{125}I (total labeled membrane protein) radioactivity. Solubilization of the PTH receptor in 1% Triton X-100 at a detergent:protein ratio of 10:1 followed by lectin affinity chromatography on WGA, S-WGA, or RCA resulted in 10.5-, 8.9-, and 8.0-fold purification, respectively (Table V). These results support the earlier findings suggesting that the order of affinity for the receptor is WGA > S-WGA > RCA_I. Passage of the once purified material over the same lectin, or chromatography over several combinations of these three lectins, did not afford any significant increase in purification.

The possibility that bPTH(1–34) covalently cross-linked to its receptor might retain its ability to bind to anti-PTH(1–34) immunoglobulin was tested initially by a standard immunoprecipitation technique (see Materials and Methods). Solubilized [^{125}I]bPTH(1–34)-labeled receptor was twice purified by WGA chromatography to remove any noncovalently bound [^{125}I]bPTH(1–34) and then immunoprecipitated with either control or immune serum. As shown in Table IV, the addition of anti-PTH IgG resulted in the precipitation of 21% of the radioactivity, while 4% was precipitated when control serum was used, and immunoprecipitation did not occur when the antibody was blocked by the inclusion of excess unlabeled bPTH(1–34). Increasing the concentration of anti-PTH(1–34) serum or the incubation time (greater than 27 h) did not result in any additional precipitation. When the immunoprecipitated material was subjected to SDS–PAGE and autoradiography, prominent labeled bands were seen at 85, 55, and <14.4 kDa, whereas substitution of normal serum for the anti-PTH(1–34) serum resulted in no detectable precipitation of the labeled receptor complex (data not shown). On the basis of these results, we attempted to purify the covalently labeled receptor on an immunoaffinity column composed of an ammonium sulfate fraction of the anti-PTH(1–34) antiserum used in the immunoprecipitation experiments. A preparation of ^{131}I/^{125}I-labeled membranes was solubilized in 1.0% Triton as previously described, except that an increased detergent:protein ratio of 100:1 (w/w) was used in an effort lessen the possibility of micelle sharing. When this preparation was chromatographed over a WGA affinity column, a 26-fold purification was achieved as shown in Table V. Application of the 26-fold purified WGA eluate to an anti-PTH(1–34) antibody affinity column provided an additional 7.8-fold purification of the PTH receptor (Table V). When this procedure was coupled with the WGA step, an overall 200-fold purification of the PTH receptor was achieved.

Discussion

Very little information is available concerning the structure of the PTH receptor. Initial results with two different photoaffinity reagents identified specifically labeled bands with M_rs of 70000 (Coltrera et al., 1981) or 60000 (Draper et al., 1982), but the concentration of unlabeled PTH(1–34) that was used to inhibit photoaffinity labeling of these components was 2–3 orders of magnitude greater than the affinity of the adenylate cyclase coupled PTH receptor in canine renal plasma membranes (Teitelbaum et al., 1982). Using the cross-linking reagent HSAB and electrolytically labeled bPTH(1–34), we have recently identified a predominant 85-kDa PTH binding protein that displays characteristics consistent with a physiologically relevant PTH receptor. Minor components are seen with M_rs of 170000, 135000, and 55000 (Nissenson et al., 1987) and <14400. Labeling of all of these components is specifically inhibited by 1 nM PTH, but not by micromolar concentrations of other peptides. Similarly, labeling is inhibited by 100 μM Gpp(NH)p or GDPβS but not by GMP or App(NH)p. These results suggest that the labeled components are constituents of PTH receptors coupled to a G-protein (presumably G_s) and are therefore physiologically relevant.

The results presented here demonstrate that the major labeled PTH receptor components in kidney are glycosylated and provide evidence for the type and partial structure of the carbohydrate components. The specific retention of the covalently labeled receptor by WGA, S-WGA, and RCA_I lectins, coupled with the altered lectin binding induced by neuraminidase treatment, strongly suggests the presence of terminal sialic acid and penultimate Gal-(β1,4)-GlcNAc. The lack of retention on Con A argues strongly against the presence of a polymannose or hybrid structure (Narasimhan et al., 1979; Debray et al., 1981, 1983; Ohyama et al., 1985). The lack of specific binding to UEA-F suggests the absence of terminal fucose residues (Debray et al., 1981, 1983). The failure to adhere to PNA, even following digestion with neuraminidase, strongly suggests the absence of terminal or penultimate Gal-(β1,3)-GalNAc (Sharon, 1975; Marshall, 1979; Sharon & Lis, 1982; Goldstein & Hayes, 1978). Taken together, these results strongly argue that the carbohydrate component of the PTH receptor is solely complex and N-linked, since few O-linked glycans lack both terminal fucose residues as well as terminal or penultimate Gal-(β1,3)-GalNAc (Sharon, 1975; Marshall, 1979; Sharon & Lis, 1982; Goldstein & Hayes, 1978).

The presence of terminal sialic acid is supported both by the increased electrophoretic mobility seen following neuraminidase treatment (Figure 3A) as well as by the differential effect of neuraminidase treatment on retention by WGA and RCA_I, with lectin binding following desialation decreasing for the former and increasing for the latter (Table II). The enhanced retention by RCA_I following treatment of the receptor with neuraminidase strongly supports this conclusion (Baenziger & Fiete, 1979).

The results with Endo F deglycosylation confirm the presence of N-linked glycans, a conclusion strongly suggested by the lectin results. Treatment of the major 85-kDa PTH receptor species with Endo F produced labeled components of 62 and 75 kDa, suggesting the presence of at least two N-linked glycan chains in the PTH receptor. It is of interest that Endo F treatment of the smaller 52–55-kDa constituent of the PTH receptor also produced two smaller forms (38 and 30 kDa). These results are consistent with the possibility that the 52–55-kDa form is a proteolytic product of the intact 85-kDa receptor and contains the sites of N-linked glycosylation. While we cannot entirely exclude the possibility that protease activity contaminating the Endo F preparation might have produced the decrease in molecular weight on SDS–PAGE, this seems unlikely. Elder and Alexander demonstrated that the only detectable protease activity in this preparation of Endo F was fully inhibitable by EDTA (Elder & Alexander, 1982); subsequent extensive use of this enzyme with multiple proteins has failed to reveal any proteolytic activity in the presence of EDTA (J. Elder, personal communication). The present experiments with Endo F were carried out in the presence of 50 mM EDTA. Additionally, we found that there was no detectable shift in the Coomassie blue staining pattern of the canine renal membrane proteins after Endo F treatment (data not shown). Thus, we tentatively conclude that the protein molecular mass of the deglycosylated, cross-linked binding component of the PTH receptor is 62 kDa. If we assume a 1:1 stoichiometry of ligand binding, the PTH receptor would have a protein molecular mass of 58 kDa.

The importance of the N-linked carbohydrate components to PTH receptor function is uncertain. Incubation of canine renal membranes with WGA markedly inhibited specific [^{125}I]bPTH(1–34) binding to its receptor. This inhibition was abolished by the presence of excess diacetylchitobiose or monomeric GlcNAc. That the presence of these saccharides does not merely enhance hormone binding in a nonspecific fashion may be seen by the absence of this effect when these sugars are incubated with S-WGA. The only other lectin that appeared to inhibit binding was RCA_I, but as this effect was seen only at an extremely high concentration of the lectin and was not reversed by coincubation with galactose, its significance is questionable. These results indicate that a terminally sialylated, complex N-linked glycan may be involved in hormone binding, may reside on the receptor in close proximity to the hormone binding site, or may mediate an allosteric effect on ligand binding. This situation is in contrast to that found with the β-adrenergic receptor. Ligand binding to the β_1-adrenergic receptor was shown to be unaffected by the presence of free lectins that bound the covalently labeled receptor (Cervantes-Olivier et al., 1985), and tunicamycin-induced inhibition of N-glycosylation was shown to have no effect on the function of β_2-adrenergic receptors (Shaji et al., 1986).

The ability to covalently label the native high-affinity PTH receptor allows quantitation of its recovery and purification without the absolute need for a functional assay. Solubilizing at a high detergent:protein ratio, we were able to achieve a 26-fold purification of the PTH receptor by lectin affinity chromatography on WGA–agarose. However, lectin chromatography has limited utility as a solitary purification step, because many membrane glycoproteins have similar terminal carbohydrate moieties (Stiles et al., 1984). The ability of anti-PTH IgG to recognize PTH that is cross-linked to its receptor could potentially provide a degree of purification that far exceeds that from lectin chromatography. The relatively poor 7.8-fold purification achieved by immunoaffinity chromatography here reflects the low 5.3% recovery from the antibody column and was due to the fact that 93% of the labeled receptor failed to bind to the column. Nevertheless, consecutive WGA and immunoaffinity chromatographic steps produced 200-fold purification of the cross-linked PTH receptor. This level of purification may prove sufficient for the production of an immunogen for the development of monoclonal antibodies against the PTH receptor.

The data presented in this paper support the view that the canine renal parathyroid hormone receptor is a glycoprotein which contains complex N-linked carbohydrate chains. We have identified carbohydrate constituents of the PTH receptor through the use of lectin affinity chromatography coupled with exoglycosidase treatment. Experiments with Endo F indicate that about 30% of the apparent mass (as defined on SDS–PAGE) of the 85-kDa PTH receptor species is carbohydrate. A possible role of this carbohydrate constituent in hormone binding is proposed, but further studies are required in order to fully characterize the functional role of this portion of the receptor. Knowledge of the carbohydrate structure permits the use of lectin chromatography as a purification procedure. Given its high recovery, this technique is especially useful as an initial purification step to remove the specifically but noncovalently bound label. The ability to immunoprecipitate the cross-linked receptor, coupled with lectin chromatography, provides the first stages of purification of this important peptide hormone receptor.

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Metabolism of 19-Methyl-Substituted Steroids by Human Placental Aromatase†

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ABSTRACT: The 19-methyl analogues of androstenedione and its aromatization intermediates (19-hydroxyandrostenedione and 19-oxoandrostenedione) were evaluated as substrates of microsomal aromatase in order to determine the effect of a 19-alkyl substituent on the enzyme's regiospecificity. Neither the androstenedione analogue [10-ethylestr-4-ene-3,17-dione (**1c**)] nor the 19-oxoandrostenedione analogue [10-acetylestr-4-ene-3,17-dione (**3c**)] was converted to estrogens or oxygenated metabolites by placental microsomes. In contrast, both analogues of 19-hydroxyandrostenedione [10-[(1*S*)-1-hydroxyethyl]estr-4-ene-3,17-dione (**2c**) and 10-[(1*R*)-1-hydroxyethyl]estr-4-ene-3,17-dione (**2e**)] were converted to the intermediate analogue **3c** in a process requiring O_2 and either NADH or NADPH. No change in enzyme regiospecificity was detected. The absolute configuration of **2e** was determined by X-ray crystallography. Experiments with $^{18}O_2$ established that **3c** generated from **2c** retained little ^{18}O (<3%), while **3c** arising from **2e** retained a significant amount of ^{18}O (≈70%). All four 19-methyl steroids elicited type I difference spectra from placental microsomes in addition to acting as competitive inhibitors of aromatase (K_I = 81 nM, 11 μM, 9.9 μM, and 150 nM for **1c**, **2c**, **2e**, and **3c**, respectively). Pretreatment of microsomes with 4-hydroxyandrostenedione (a suicide inactivator of aromatase) abolished the metabolism of **2c** and **2e** to **3c**, as well as the type I difference spectrum elicited by **2c** and **2e**. The failure of **2c**, **2e**, and **3c** to undergo aromatization was rationalized in the context of a mechanistic proposal for the third oxygenation of aromatase requiring hydrogen abstraction at C_1 of 19,19-dihydroxyandrostenedione, homolytic cleavage of the C_{10}–C_{19} bond, and oxygen rebound at C_{19}.

Aromatase is a microsomal P-450 that executes three successive monooxygenations on androstenedione (**1a**) to generate in order 19-hydroxyandrostenedione (**2a**), 19-oxoandrostenedione (**3a**), and the products estrone (**4a**) and formic acid (Meyer, 1955; Ryan, 1959; Longchampt et al., 1960; Morato et al., 1961; Wilcox & Engel, 1965; Akhtar & Skinner, 1968; Axelrod et al., 1965; Starka & Breuer, 1970; Thompson & Siiteri, 1974a,b). An analogue of androstenedione in which a C_{19} hydrogen has been replaced by an acetylene (**1b**) acts as a suicide inactivator of aromatase (Covey et al., 1981; Metcalf et al., 1981; Marcotte & Robinson, 1982), although its mechanism in doing so is unknown. By executing its normal catalytic function, aromatase could generate an acetylenic ketone via the sequence **1b** → **2b** → **3b**. Michael addition of an enzyme nucleophile to **3b** would result in the formation of

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