SOLUBILIZATION OF A PARATHYROID HORMONE RECEPTOR FROM BOVINE KIDNEY CORTEX PLASMA MEMBRANES

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SUMMARY: Preparation of a soluble extract of bovine kidney cortex plasma membranes with Triton X-100 is described. Interaction of this extract with tritiated parathyroid hormone was detected using three distinct binding assays: gel filtration chromatography, precipitation of bound hormone with polyethylene glycol, and adsorption of free hormone with microfine silica. The binding of parathyroid hormone to the component(s) in the extract was highly specific and sensitive to competition by purified native parathyroid hormone and synthetic (1-34) bovine parathyroid hormone. Inactivation of the tritiated parathyroid hormone by oxidation led to loss of its binding activity.

Understanding polypeptide hormone action at the molecular level depends on identification, extraction, isolation and characterization of the cellular structure(s) with which the hormone initially interacts, i.e., a receptor. For parathyroid hormone (PTH)* the existence of specific receptors in a bovine kidney plasma membrane preparation was first demonstrated by Sutcliffe et al. (1) and later shown by Malbon and Zull (2) in rat kidney plasma membranes, and by DiBella et al. again in bovine membranes (3). In this communication we now present data which suggest further progress toward these long-range goals; i.e., the solubilization of PTH receptor from kidney membrane preparations.

MATERIALS AND METHODS. Highly purified, bovine parathyroid hormone (1800-2200 Units/mg protein) was purchased from Inolex Pharmaceuticals, Glenwood, Ill.

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*Abbreviations used are: PTH, parathyroid hormone; [^{3}H]PTH, tritiated acetamidino parathyroid hormone; ACTH, adrenocorticotropic hormone, OX-[^{3}H]PTH, oxidized, tritiated acetamidino parathyroid hormone; EDTA, ethylenediamine tetraacetic acid; PEG, polyethylene glycol; BSA, bovine serum albumin.

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Bovine insulin, glucagon and lysine-vasopressin were purchased from Sigma. Synthetic human ACTH was a gift from Dr. George Sayers, Dept. of Physiology, Case Western Reserve University, Cleveland, Ohio. Bio-Bead SM-2 and Bio-Gel P-100 were purchased from Bio-Rad Laboratories, Rockville Centre, N. Y. Triton X-100 was purchased from RPI Corporation, Elk Grove Village, Ill. Quso G-32 was purchased from Philadelphia Quartz Company, Chester, Penn. All other chemicals were obtained from Fisher Scientific, Fair Lawn, N. J.

Details of the preparation of the tritiated derivative of PTH, [^{3}H] acetamidino PTH, its biologically inactive, peroxidized counterpart OX-[^{3}H]PTH, and characterization of this tracer has been reported elsewhere (4, 5). The specific activity of the hormone used in these studies was 4.8 x 10^6dpm per microgram.

Preparation of a soluble extract of partially purified plasma membranes of bovine kidney cortex is described below. Bovine kidney cortex fresh from slaughter was processed to the point of partially purified membranes as detailed previously (6). This partially purified membrane fraction was washed free of sucrose and suspended in a 25mM Tris-HCl, pH 7.5, 1mM EDTA buffer with a final protein concentration of 2-4 mg/ml. This suspension was diluted with an equal volume of a 2% Triton X-100 (w/w), 25mM Tris-HCl, pH 7.5, 1mM EDTA buffer and homogenized at 0^oC with two strokes of a Potter-Elvehjem homogenizer. The mixture was vigorously shaken at 2^oC for 2 hours and then centrifuged at 100,000 x G_{max} for 2 hours in a Beckman L-50 ultracentrifuge fitted with a type 50 rotor set at 4^oC. The resultant pellet was discarded and the supernatant consisting of solubilized material (i.e., "the extract") was aliquoted and stored at -20^oC.

The detergent was removed from the extract by vigorous agitation with Bio-Bead SM-2 following the procedure of Holloway (7). The protein concentration of the extract was determined by the method of Lowry et al. (8), using bovine serum albumin as a standard. Details of this procedure will be given in a subsequent publication (9).

Three distinct binding assays were employed: (a) gel filtration column chromatography of incubation mixtures containing extract and [^{3}H]PTH on Bio-Gel P-100 columns which allow distinct separation of free and bound PTH; (b) precipitation of macromolecular-bound [^{3}H]PTH from solution with polyethylene glycol (10); and (c) adsorption of the free [^{3}H]PTH from solution with microfine silica, Quso G-32 (11). The details of each of these methods are given in the following section.

Fractions from the gel filtration columns, pellets from the polyethylene glycol precipitation, and aliquots of the supernatants before and after Quso G-32 treatments were solubilized with NCS (Amersham/Searle) and counted in a Picker Nuclear Liquimat. The efficiency of the counting was established and results converted to dpm.

RESULTS. As shown in Figure 1, incubation of the kidney extract with [^{3}H]PTH followed by chromatography of the mixture on Bio-Gel P-100 showed distinct evidence of an interaction between the hormone and a macromolecule. Whereas the hormone alone elutes as a single peak at the position indicated midway between the void and salt volumes, the mixture chromatographed as two major peaks, one eluting in the void volume and representing 30-40% of the total, and the second eluting in the position expected for free PTH. A third smaller peak eluting in the salt volume suggests that some degradation of the hormone may also have occurred during the incubation. Preincubation of the extract with a 100-fold molar excess of unlabeled, native PTH significantly reduced the subsequent binding of the [^{3}H]PTH to the extract. This observation suggests a binding process with some specificity and reduces the probability that the observed binding is artifactual. The presence of the unlabeled, native PTH did not affect the third peak in any way.

In view of the importance of the above conclusions we sought further evidence by two completely independent means: (a) precipitation of the receptor-PTH complex with polyethylene glycol and, (b) removal of unbound [^{3}H]PTH from solution by adsorption on microfine silica (Quso G-32). Figure 2 demonstrates

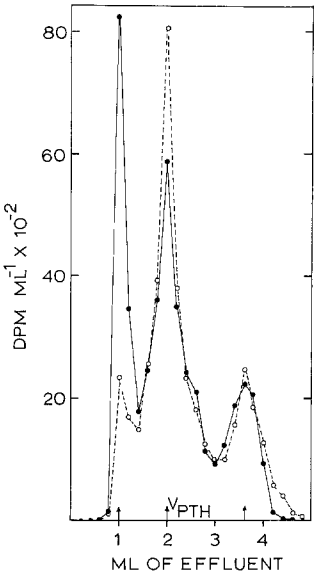

Figure 1. Bio-Gel P-100 gel filtration chromatography of 0.5 mg of bovine kidney cortex soluble extract incubated with 10 ng of [^{3}H]PTH preincubated 1 hr at 0°C with (O---O) and without (●——●) 1 µg of unlabeled native PTH. Extract and [^{3}H]PTH were incubated at 4°C for 12 hr in a 0.1% BSA, 25mM Tris-HCl, pH 7.5 1mM EDTA buffer at a final volume of 100 µℓ. Chromatography performed in 0.5 M Tris-HCl pH 7.5 on 26 x 0.4 cm lucite columns of Bio-Gel P-100 (50-100 mesh). Void volume and salt volume are indicated by arrows on the chromatograph.

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the ability of polyethylene glycol to precipitate macromolecular bound [^{3}H]PTH from a common incubation mixture containing extract and the labeled hormone. The minimum final concentration of PEG required to assure maximum precipitation of macromolecular-bound [^{3}H]PTH was 12% w/w. Under the conditions specified in the legend of Fig. 2 this concentration of PEG precipitates up to 40% of the radioactivity from the [^{3}H]PTH-extract mixture, whereas under identical conditions in an incubation containing only the labeled hormone and no extract, only 3-4% of the radioactivity is precipitated.

Table I summarizes the results of further studies utilizing the PEG methodology. Preincubation with unlabeled, native PTH significantly reduces the subsequent binding of the [^{3}H]PTH to the solubilized components precipitated by PEG. However, similar preincubations with very large amounts of ACTH, in-

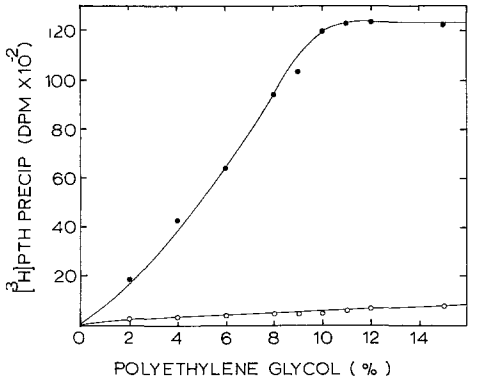

Figure 2. Precipitation of bound PTH by increasing concentration of polyethylene glycol. 150 mg of protein extract was incubated for 12 hr at 4°C with 8 ng of tritiated PTH in 0.1% BSA, 25mM Tris, 1mM EDTA, pH 7.5 in 400µℓ microfuge tubes. At the end of the incubation period 100 µℓ of chilled phosphate buffer (0.1 M) pH 7.5 containing 0.1% human gammaglobulin was added and the tubes placed on ice. 200 µℓ of the same phosphate buffer containing PEG to give a final concentration indicated was then added, agitated and allowed to stand on ice for 20 minutes. The tubes were then centrifuged at 10,000 x G_{max} for 10 min at 4°C. The supernatants were aspirated and the tip of the microfuge tube containing the pellet was clipped blotted on filter paper and placed in scintillation vials. For incubations without extract the hormone was incubated with serum albumin alone. PEG precipitations with (●—●) and without (○—○) soluble extract present.

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sulin, glucagon and vasopressin fail to reduce the subsequent binding of [^{3}H]PTH. Synthetic (1-34) bovine PTH was also competitive with the binding of tritiated native PTH, but was not as effective on a molar basis as the native PTH. Inactivation of [^{3}H]PTH by oxidation with H_2O_2 led to a reduction in binding, shown also in Table I. This reduced level of bound radioactivity furthermore displays no sensitivity to competition by active, unlabeled PTH.

Finally, as indicated in Table II, assays of the radioactivity remaining in solution after addition of Quso G-32 to an extract-[^{3}H]PTH mixture, gave qualitatively similar results. Approximately 9% of the radioactivity present in the incubations for these experiments was not precipitated by Quso G-32 in the absence of any kidney extract, but more than 25% of the radioactivity remained in solution when the extract was present. The reduction of "receptor bound" radioactivity by preincubation with unlabeled native PTH was almost to-

TABLE I

Specificity of [^{3}H]PTH Binding To Soluble Extract

Tritiated hormone was incubated as described for Fig. 2 with preincubations of 1 hour with competing hormone where competition was investigated. At the end of incubation, the soluble extract was precipitated with a final concentration of 12% PEG by the procedure described in Fig. 2. Experiments A utilized [^{3}H]PTH, experiments B utilized the oxidized tritiated hormone in addition.

Experiment	Radioactive Hormone	Competitor	Competitor Concentration(nM)	Binding(dpm)*	% Inhibition
A	[^{3}H]PTH	None	----	7012 ± 206	----
	"	PTH	526	3902 ± 156	45
	"	PTH	1000	3182 ± 282	56
	"	1-34 PTH (synthetic)	1000	5400 ± 158	23
	"	ACTH	2180	7088 ± 154	0
	"	Glucagon	2850	6752 ± 166	4
	"	Insulin	1720	6698 ± 180	4
	"	Vasopressin	10,000	6722 ± 90	4
B	[^{3}H]PTH	None	----	7012 ± 206	----
	OX[^{3}H]PTH	None	----	2068 ± 246	----
	[^{3}H]PTH	PTH	1000	3182 ± 282	56
	OX[^{3}H]PTH	PTH	1050	1932 ± 31	6

* Results expressed as dpm ± S.E.M. of five determinations.

TABLE II

Quso G-32 Assay of [^{3}H]PTH Binding To Soluble Extract

200 µℓ incubations containing 335 µg protein of soluble extract and 10 ng tritiated PTH in same buffer used for Table I were incubated 60 min at 4°C. 0.5 mg of Quso G-32 was then added, agitated vigorously, and pellet by 10 min centrifugation in Beckman 152 microfuge. The incubation medium was multi-aliquoted before and after Quso treatment. Competition studies were performed with 10 min pre-incubations with 500 fold molar excess of highly purified PTH at 4°C prior to addition of radioactive hormone. Results reported in dpm ± S.E.M.

Addition*	dpm in Supernatant	Receptor Bound dpm**
a. 1% BSA b. [^{3}H]PTH	5712 ± 76	
a. soluble extract b. [^{3}H]PTH	14114 ± 232	8402
a. soluble extract b. 500nM unlabeled PTH c. [^{3}H]PTH	5824 ± 1028	112

* Listed in order of addition to incubation vessel.
** Dpm in supernatant minus dpm not bound by Quso-treatment

tal, again showing competition of unlabeled, native PTH with the tritiated hormone for the solubilized receptor.

DISCUSSION. From the data obtained in these experiments it is clear that Triton X-100 treatment of beef kidney membranes leads to solubilization of a component with the capacity to bind parathyroid hormone. The likelihood that this observation is artifactual is greatly reduced by the fact that the same conclusion is obtained by three separate techniques, gel filtration chromatography, PEG precipitation of bound hormone, and Quso G-32 binding of free hormone. That the solubilized component has specificity, is sensitive to competition by cold PTH as well as an active peptide fragment derived from PTH (1-34, synthetic bovine PTH) and does not show specific binding of the inactive oxidized form of PTH all adds further support to this fundamental conclusion.

This putative receptor appears to be a macromolecule of molecular weight larger than 100,000 as evidenced by its elution position in Bio-Gel P-100, and since it remains soluble after removal of the detergent further characterization should be possible. However, only more extensive careful study will reveal whether this solubilized component is in fact, as we suggest here, a physiologically important membrane receptor for PTH. Such study is presently underway.

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