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A SPECIFIC, HIGH AFFINITY BINDING PROTEIN FOR 1α,25-DIHYDROXY VITAMIN D IN THE CHICK OVIDUCT SHELL GLAND

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SUMMARY: A binding activity for the steroid hormone 1,25-dihydroxy vitamin D was observed in partially purified cytosol extracts of oviduct shell gland from vitamin D-deficient chicks. Binding is a saturable, high affinity (K_D = 0.13 nM) process which is N-ethyl maleimide-sensitive and specific for 1,25-dihydroxy vitamin D over 25-hydroxy vitamin D. The 1,25-dihydroxy vitamin D-binding activity sediments at 3.7 S in sucrose density gradients and binds to DNA-cellulose columns, eluting as a single peak at 0.21 M KCl. These properties, along with a preferential localization of binding activity in the shell gland region of the oviduct, are consistent with the identification of this 1,25-dihydroxy vitamin D-binding protein as a steroid hormone receptor involved in the regulation of egg shell calcification by vitamin D.

INTRODUCTION

The active metabolite of vitamin D, 1,25-dihydroxy vitamin D_3 (1,25-$(OH)_2D_3$) is a steroid hormone which plays a major role in the regulation of calcium metabolism, primarily through its effects on intestine and bone (1,2). One of the most specialized examples of calcium metabolism occurs in the domestic chicken, where approximately 2 g of calcium per day are utilized in the mineralization of the egg shell (3). Vitamin D is required for this process: hens maintained on a vitamin D-deficient diet lay fewer eggs with thin shells, and subsequent vitamin D administration restores normal egg production (4). Furthermore, estrogen administration to non-laying birds increases kidney 25-OH D_3 1α-hydroxylase levels (5,6), and in the laying hen, circulating 1,25-$(OH)_2D_3$ levels peak daily at the time of maximal shell calcification (7). This stimulation of 1,25-$(OH)_2D_3$ production is presumably required for increased intestinal calcium absorption during egg laying as well as for mobilization of medullary bone (8); but it also may be directly involved in the mineralization of the egg shell. The present investigation provides evidence for the direct

Abbreviations used: 1,25-$(OH)_2D_3$, 1α,25-dihydroxy vitamin D_3; 25-OH D_3, 25-hydroxy vitamin D_3; NEM, N-ethylmaleimide.

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role of vitamin D in egg shell calcification with the identification of a chicken oviduct shell gland 1,25-$(OH)_2D_3$ binding protein whose properties are characteristic of a steroid hormone receptor.

MATERIALS AND METHODS

Female white leghorn chicks (Pacesetter Farms, Alta Loma, CA) were raised from day 1 after hatching on a vitamin D-deficient diet (9), and oviduct growth was induced by injection with diethylstibestrol (20 mg) in polyethylene glycol paste at 1 and 3 weeks after hatching (10). [^{3}H]1,25-$(OH)_2D_3$ was prepared enzymatically (11) from [^{3}H]25-OH D_3 (Amersham-Searle, 11.7 Ci/mmole) using a kidney homogenate from vitamin D-deficient male white leghorn chicks and purified by silicic acid, Sephadex LH-20 and celite liquid-liquid partition chromatography (12) prior to use in binding studies. Unlabelled vitamin D derivatives (obtained from Dr. M.R. Uskokovic, Hoffmann-LaRoche) were purified by celite chromatography (13) and analyzed by UV absorbance.

Cytosol fractions from tissues of 4 to 6 week old chicks were prepared as follows (all steps were carried out at 0-4°): Tissues were homogenized in 2.5 volumes (weight to volume) of 0.25 M sucrose, 50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, 12 mM monothioglycerol, pH 7.4 (buffer 1) by 4 strokes of a motor-driven (600 RPM) teflon-glass homogenizer (for intestine, kidney and liver) or 3 10 sec bursts of a Polytron PT10 ST tissue disruptor (Brinkmann Instruments) at a setting of 6 (for oviduct shell gland, isthmus and magnum). Homogenates were centrifuged for 10 min at 12,000 x g and then 1 hr at 150,000 x g, the floating fat layer removed by aspiration, and the clear supernatant fraction was designated cytosol. Ammonium sulfate fractionation of cytosols was carried out by the addition of solid $(NH_4)_2SO_4$ to 35% saturation, followed by centrifugation for 10 min at 12,000 x g. The pellet fractions were redisolved in 10 mM Tris-HCl, 1 mM EDTA, 12 mM monothioglycerol (buffer 2) plus 0.15 M KCl for use in binding studies.

Binding of vitamin D metabolites was initiated by the addition of cytosol or ammonium sulfate precipitate fractions to sterols dissolved in ethanol. After incubation at 0-4°, binding was analyzed by sucrose gradient sedimentation, hydroxylapatite absorption or column chromatography. Sucrose gradient centrifugation was carried out by layering 200 µl samples on linear gradients of 6-22% (cytosol) or 4-20% (ammonium sulfate precipitate) sucrose (weight to volume) in buffer 2 plus 0.15 M KCl, followed by centrifugation for 18 hrs at 216,000 x g. Gradients were fractionated into 7 drop fractions, and radioactivity determined by scintillation counting. ^{14}C-labelled marker proteins ovalbumin (3.7 S) and γ-globulin (7 S) were prepared by the method of Means and Feeney (14), and were included as internal standards in some sucrose gradient samples. Hydroxylapatite assay of bound sterol was carried out by the method of Wecksler and Norman (15), and DNA-cellulose and DEAE-cellulose chromatography was performed as previously described (10).

Protein was determined by the methods of Lowry et al. (16) and Bradford (17) with comparable results.

RESULTS

Sucrose gradient velocity sedimentation previously has been used to identify vitamin D receptors in cytosol preparations from intestine (18-20), parathyroid gland (21), kidney and pancreas (22) and bone (23). This procedure

allowed the separation of radioactive hormone bound to receptors from these tissues, sedimenting at approximately 3 to 4 S, from unbound sterol and from [^{3}H]1,25-(OH)$_2$D$_3$ bound to a 6 S complex of serum 25-OH D$_3$ binding protein and unknown cytosol proteins (24). When we employ this procedure to analyze chick shell gland cytosol for 1,25-(OH)$_2$D$_3$ binding, very low levels of a complex sedimenting at approximately 3.7 S are observed (Fig. 1A). However, most of the radioactive hormone was bound to the 6 S complex, which is present in much larger amounts in shell gland than in intestinal cytosol. To reduce this interference and improve resolution of vitamin D-binding activities, shell gland cytosol was fractionated by precipitation with ammonium sulfate.

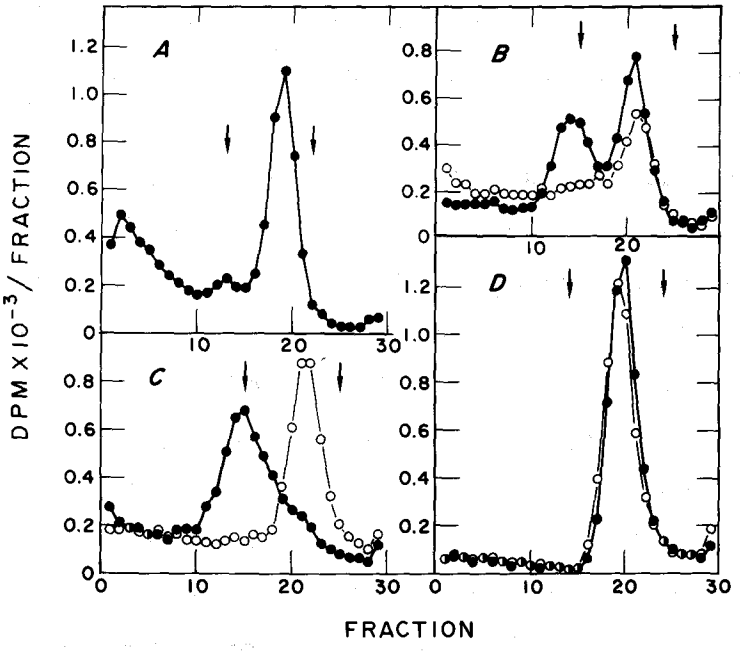

Fig. 1. Sucrose gradient sedimentation analysis of vitamin D metabolite binding by chick shell gland cytosol and ammonium sulfate precipitate fractions. A. Cytosol binding of [^{3}H]1,25-(OH)$_2$D$_3$. B. Ammonium sulfate precipitate binding of [^{3}H]1,25-(OH)$_2$D$_3$ in the absence (•) or presence (o) of a 100-fold excess of unlabelled 1,25-(OH)$_2$D$_3$. C. Ammonium sulfate precipitate binding of [^{3}H]1,25-(OH)$_2$D$_3$ in the presence of a 100-fold excess of unlabelled 25-OH D$_3$ (•) or after pretreatment for 10 min at 0° with 20 mM NEM (o). D. Ammonium sulfate precipitate binding of [^{3}H]25-OH D$_3$ before (•) or after (o) pretreatment with NEM. Sedimentation is from left to right, and arrows indicate the sedimentation positions of internal marker ^{14}C-labelled ovalbumin (3.7 S) and γ-globulin (7 S). Samples were incubated 2 hr at 0° with 2 nM radioactive sterol with or without 200 nM unlabelled sterol in the presence of 4% ethanol prior to density gradient centrifugation.

Analysis of [^{3}H]1,25-$(OH)_2D_3$ binding by the shell gland ammonium sulfate precipitate (Fig. 1B) reveals a much greater proportion of bound hormone sedimenting at 3.7 S than at 6 S compared to cytosol, and these two binding activities can be distinguished by their sterol binding specificities. The 3.7 S activity is abolished by addition of unlabelled 1,25-$(OH)_2D_3$ in 100-fold excess (Fig. 1B, o), but not by addition of a similar amount of unlabelled 25-OH D_3 (Fig. 1C, •). In contrast, the 6 S binding activity is only partially diminished by unlabelled 1,25-$(OH)_2D_3$, whereas it is completely displaced by unlabelled 25-OH D_3. Also, [^{3}H]25-OH D_3 is bound only by the 6 S protein; no 3.7 S 25-OH D_3 binding activity is observed (Fig. 1D, •). The sterol binding specificity of the 3.7 S activity is comparable to that seen with vitamin D receptors from other chick and rat tissues (18-23,25,26).

These two shell gland vitamin D-binding activities can also be distinguished by their sensitivity to sulfhydryl-blocking reagents. N-ethylmaleimide (NEM) pretreatment inhibits [^{3}H]1,25-$(OH)_2D_3$ binding by the 3.7 S activity (Fig. 1C, o), while 6 S binding of [^{3}H]1,25-$(OH)_2D_3$ and [^{3}H]25-OH D_3 (Fig. 1D, o) are unaffected. Sensitivity of hormone binding to sulfhydryl blocking reagents is a characteristic common to many steroid hormone receptors (27-29).

Unfilled 1,25-$(OH)_2D_3$ binding sites in shell gland cytosol decay rapidly, precluding equilibrium binding analysis. However, $(NH_4)_2SO_4$ precipitation stabilizes unfilled sites for 24 hours at 0° (data not shown), and [^{3}H]-1,25-$(OH)_2D_3$ binding can be characterized using a hydroxylapatite batch procedure (15). Binding is saturable (Fig. 2) and is inhibited either by pretreatment with NEM or addition of excess unlabelled 1,25-$(OH)_2D_3$. When the NEM-sensitive binding component is analyzed by the method of Scatchard (30) (inset), an apparent K_D of 0.13 nM is observed, with a binding site concentration in this preparation of 0.057 pmoles/mg protein. The amount of NEM-sensitive 1,25-$(OH)_2D_3$ binding determined by the hydroxylapatite assay is consistent with the amount of 3.7 S binding component observed in sucrose gradient analysis using several preparations of shell gland ammonium sulfate precipitate.

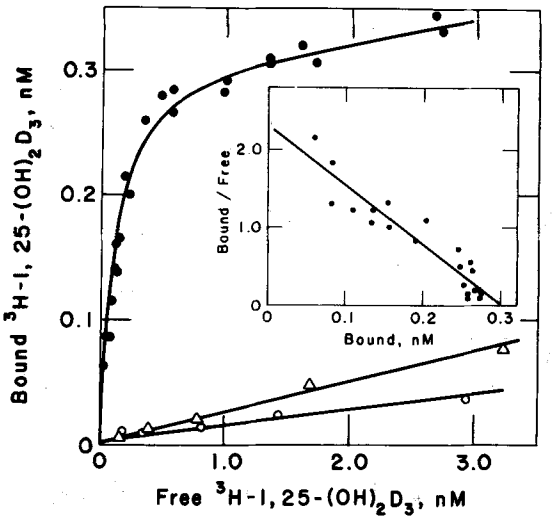

Fig. 2. Saturation of [^{3}H]1,25-(OH)$_2$D$_3$ binding to shell gland $(NH_4)_2SO_4$-precipitated cytosol. Protein samples (0.25 ml) were incubated with [^{3}H]1,25-(OH)$_2$D$_3$ at varying concentrations for 16 hr at 0° in the presence of 10% ethanol (•). Total [^{3}H]1,25-(OH)$_2$D$_3$ was determined on 0.05 ml aliquots of each sample, and bound hormone was determined on the remainder by hydroxylapatite absorption. Control samples were assayed for sterol binding after pretreatment with 12.5 mM NEM (Δ) or in the presence of a 100-fold excess of unlabelled 1,25-(OH)$_2$D$_3$ (o). Inset: Scatchard plot of the NEM-sensitive [^{3}H]1,25-(OH)$_2$D$_3$ binding.

These results are consistent with the identification of the shell gland 3.7 S 1,25-(OH)$_2$D$_3$ binding activity as a vitamin D receptor. One characteristic common to all steroid hormone receptors is their ability to bind to nuclei and nuclear components (DNA, chromatin) both *in vivo* and *in vitro* (31,32). Therefore, the shell gland 1,25-(OH)$_2$D$_3$ binding activity was examined for its ability to bind to DNA using affinity chromatography on DNA-cellulose. When shell gland ammonium sulfate precipitate is complexed with [^{3}H] 1,25-(OH)$_2$D$_3$ and then applied to a DNA-cellulose column, the 1,25-(OH)$_2$D$_3$ binding activity is almost completely (> 90%) retained, and can be eluted with a linear KCl gradient as a single symmetrical peak at about 0.21 M KCl (Fig. 3A) in about 50 to 80% yield. This binding is not seen in the presence of excess unlabelled 1,25-(OH)$_2$D$_3$ or after prior treatment with NEM, but is only slightly reduced by excess unlabelled 25-OH D$_3$. In contrast, the [^{3}H] 25-OH D$_3$ binding activity in the same preparation is completely unretarded by DNA-cellulose and subsequently binds to DEAE-cellulose and elutes with a linear KCl gradient as a single peak at approximately 0.13 M KCl (Fig. 3B).

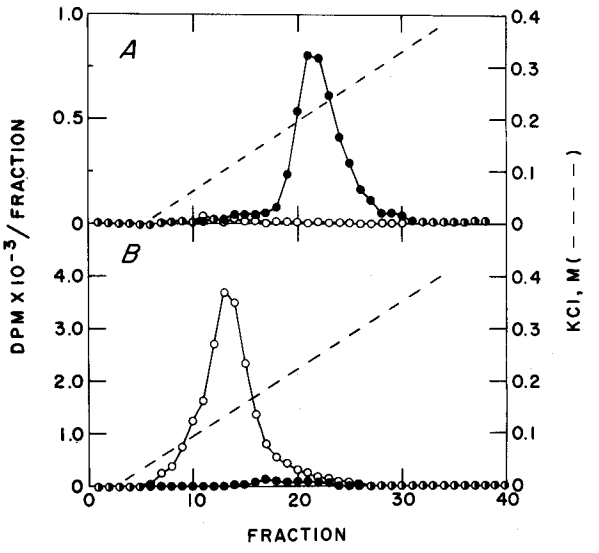

Fig. 3. Sequential DNA-cellulose and DEAE-cellulose column chromatography of shell gland vitamin D metabolite binding activities. Shell gland $(NH_4)_2SO_4$-precipitated cytosol (0.375 ml) was incubated with 5 nM [^{3}H]1,25-$(OH)_2D_3$ (•) or [^{3}H]25-OH D_3 (o) for 16 hr at 0° in buffer 2 plus 0.15 M KCl. Samples were then diluted with 4 ml 10 mM Tris-HCl, 0.5% triton X-100, pH 7.4 and applied to 1 ml DNA-cellulose columns. The unabsorbed material and subsequent wash fractions were passed through a 1 ml DEAE-cellulose column. The columns were washed with 6 ml 10 mM Tris-HCl, 0.5% triton X-100, pH 7.4, and then with 10 ml buffer 2. The columns were disconnected, and each column was eluted with a 40 ml gradient of 0 to 0.5 M KCl in buffer 2. Fractions of 1 ml were collected and analyzed for KCl (conductivity) and radioactivity.

The oviduct is a highly specialized organ which can be separated both functionally and anatomically into distinct regions (33), including the magnum (egg white secretion), isthmus (shell membrane formation) and shell gland (fluid secretion and calcification). These separate regions were examined for the presence of 1,25-$(OH)_2D_3$ binding activity. As shown in Table 1, there is binding activity in all three regions; however, the specific activity is approximately 3-fold higher in the shell gland than in the isthmus or magnum. For comparison, the levels in parallel preparations from intestine, kidney and liver are shown; of these tissues, the shell gland has the second highest binding activity. The values of binding activity for isthmus, magnum and kidney are significant; small but reproducible peaks of activity which are both 1,25-$(OH)_2D_3$-displaceable and sensitive to NEM can be resolved by DNA-cellulose chromatography (not shown).

Table 1. Tissue distribution of 1,25-$(OH)_2D_3$ binding activity

Tissue	Specific [^{3}H]1,25-$(OH)_2D_3$ binding* pmoles/g tissue	pmoles/mg protein
Shell gland	0.53	0.13
Oviduct isthmus	0.17	0.038
Oviduct magnum	0.19	0.036
Small intestine	1.5	0.40
Kidney	0.17	0.038
Liver	0.04	0.007

*Ammonium sulfate-precipitated cytosol fractions were incubated for 16 hrs at 0° with 2 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence of 10% ethanol. Binding was measured by hydroxylapatite absorption, and values were corrected for bound sterol in samples pretreated with 10 mM NEM.

DISCUSSION

These results clearly identify a shell gland 1,25-$(OH)_2D_3$ binding protein which has the characteristics of a steroid hormone receptor. Recent studies have shown marked elevation of circulating 1,25-$(OH)_2D_3$ levels in birds upon estrogen administration (5,6) and during egg laying (7), which leads to increased intestinal absorption of calcium and resorption of medullary bone to meet the metabolic demand of egg production. Our finding of preferential localization of a 1,25-$(OH)_2D_3$ binding protein in the oviduct shell gland suggests an additional direct function of vitamin D in the regulation of egg shell calcification. Thus the shell gland, an estrogen-dependent reproductive organ, should prove a useful system to study both the regulation of mineral deposition by vitamin D and the effects of reproductive hormones on this process.

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