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STUDIES ON THE MODE OF ACTION OF CALCIFEROL XXXII. EVIDENCE FOR A 24(R),25(OH)$_2$-VITAMIN D$_3$ RECEPTOR IN THE PARATHYROID GLAND OF THE RACHITIC CHICK[#]

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Summary: Previous studies from a number of laboratories suggest a biological role for 24(R),25-dihydroxyvitamin D$_3$ [24,25(OH)$_2$D$_3$] in bone mineralization, parathyroid hormone secretion and possibly intestinal calcium absorption. As a consequence we have initiated a search for a binding protein/receptor for 24,25(OH)$_2$D$_3$ analogous to that previously shown to exist in many tissues for 1,25-dihydroxyvitamin D$_3$. Here we report for the first time the existence of a 24,25(OH)$_2$D$_3$ receptor/binding protein in the parathyroid glands of rachitic chicks. Incubation of a 105,000 x g supernatant of a 0.4 M KCl extract of parathyroid gland chromatin with [^{3}H]-24,25(OH)$_2$D$_3$ both before and after 5-20% sucrose density gradient centrifugation resulted in the detection of a receptor-like binding component with a mobility of 3.2-3.4 S. This putative 24,25(OH)$_2$D$_3$ receptor was shown to have ligand specificity for 24,25(OH)$_2$D$_3$ over 25-hydroxyvitamin D$_3$ and to be a different macromolecular species than the 6 S binding protein for 25(OH)D$_3$ which is present in the cytosol but not chromatin of the parathyroid gland.

Introduction: The seco-steroid vitamin D$_3$ is metabolized into two biologically active, dihydroxylated metabolites; namely 1,25(OH)$_2$D$_3$[1] which has been extensively studied (2) and 24,25(OH)$_2$D$_3$[1] which has until recently received relatively little attention (3). Several reports have appeared which suggest that 24,25(OH)$_2$D$_3$ has possible physiological functions distinct and different from that of [1,25(OH)$_2$D$_3$]. Studies in normal dogs and dogs

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[#]The previous paper in this series is reference (1).

[1]The abbreviations used are: 25(OH)D$_3$ (25-hydroxycholecalciferol); 1,25-(OH)$_2$D$_3$ (1α,25-dihydroxycholecalciferol); 24,25(OH)$_2$D$_3$ [24(R),25-dihydroxycholecalciferol]; HAP (hydroxylapatite); TED (10 mM Tris-HCl, 1.5 mM EDTA [ethylenediamine tetraacetate], 1 mM DTT [dithiothreitol] pH 7.4, 4°C), KTED (TED in 0.3 to 0.4 M KCl); PBD (scintillation cocktail: 5 g of 2-(4'-tertiarybutylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole in 1 L toluene); PTG (parathyroid gland); SDGA (sucrose density gradient analysis); [^{14}C]-BSA ([^{14}C] labelled bovine serum albumin).

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with experimentally induced renal hypoparathyroidism showed a reduction in parathyroid hormone (PTH) secretion after both acute (4) and chronic (5) $24,25(OH)_2D_3$ application. Another study in chicks with hyperparathyroidism secondary to rickets showed that the combined presence of $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were required to effect reversal of the PTG hypertrophy (6). Similarly, Henry and Norman (7) found that both dihydroxylated vitamin D metabolites were required for normal egg hatchability; the absence of either $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ resulted in a failure of fertile eggs to hatch. Also a number of other reports have appeared (8-12) which support the existence of unique biological effects for $24,25(OH)_2D_3$. Assuming that $24,25-(OH)_2D_3$ functions in these systems in an analogous steroid hormone-like fashion to that proposed for $1,25(OH)_2D_3$, then a key supportive observation would be demonstration of a specific binding protein/receptor for $24,25(OH)_2-D_3$. Accordingly a biological response to $24,25(OH)_2D_3$ in a target tissue would be mediated, in part, by formation of a specific ligand-receptor complex. This steroid-receptor complex would then migrate to the nucleus and initiate the synthesis of specific messenger RNA(s). Therefore we have studied by sucrose density gradient analysis the cytosol and chromatin fractions of one of the most likely target tissues for $24,25(OH)_2D_3$ receptor content, namely the parathyroid gland.

METHODS

Animals: White leghorn cockerels were obtained on the day of hatch (Pace/Setter, Alta Loma, California). They were fed a standard chick starter mash (Poultrymen's Cooperative Association, California) (0.6% calcium, 0.4% phosphorus) for the first two weeks. They were then raised for an additional ten weeks on our standard vitamin D-deficient rachitogenic diet (13). This protocol resulted in birds possessing parathyroid glands (PTG) which had undergone marked hypertrophy. The PTG from these animals weigh approximately 40-60 mg. Normally fifteen birds were sacrificed and the dissected PTG immediately collected in an ice cold TED buffer. The PTG were then homogenized in 4 volumes (20%) of TED with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 500 x g for 10 min at 4°C to yield a nuclear pellet and a crude cytosol supernatant fraction. A purified cytosol fraction was prepared by centrifugation at 105,000 x g for 60 min at 4°C. It was essential to keep the cytosol at all times at 4°C.

Crude parathyroid gland chromatin was prepared at 4°C by resuspending the above nuclear pellet three times in TED 0.5% Triton X-100 (pH = 7.4) followed by centrifugation at 10,000 x g for 10 min. The resulting crude chromatin pellet was then extracted in 0.4 M KCl-TED (same volume as homogenization buffer) for 45 min with frequent mixing. The chromatin extract was centrifuged (5,000 x g/10 min) and the resulting supernatant centrifuged at 105,000 x g for 1 hour.

Sucrose density gradient analysis: Linear 5-20% sucrose density gradients in TED (4 ml) were made using a peristalic pump and gradient former (Buchler Instruments, Switzerland). Samples (0.2 ml) were carefully layered on top of the gradients which had been pre-equilibrated for 5 hours at 4°C. The gradients were centrifuged for 21.5 hours at 4°C in a SW-60 rotor (Beckman Instruments) at 50,000 rpm and 5 drop fractions collected. The sedimentation rates (S) for standard proteins were calculated from a standard [^{14}C]-labeled bovine serum albumin (S = 4.4) or ovalbumin (S = 3.7).

Hydroxylapatite Assay: Receptor-bound tritiated vitamin D metabolites were detected in sucrose density gradient fractions by hydroxylapatite (HAP) assay (14). A 0.5 ml aliquot of 50% (by volume) HAP slurry at 4°C was added to the fractions for a 15 min incubation with frequent vortexing. The samples were then centrifuged at 12,000 x g for 5 min. The HAP pellets were washed three times with 2 ml TED 0.5% Triton X-100 (pH = 7.4). The final washed pellet was extracted for tritiated steroids with ethanol. The dried extracts were dissolved in PBD for radioactivity quantification by liquid scintillation counting.

Chemicals: [^{3}H-23,24(n)]-25(OH)D_3 (110 Ci/mmol), [^{3}H-23,24(n)]-24(R)-25(OH)$_2D_3$ (68 Ci/mmol), [^{3}H-23,24(n)]-1α,25(OH)$_2D_3$ (110 Ci/mmol) were obtained from Amersham/Searle (Arlington Heights, IL). Unlabeled 25(OH)D_3, 24(R),25(OH)$_2D_3$ and 1α,25(OH)$_2D_3$ were the kind gifts of Dr. Milan Uskokovič, Hoffmann-La Roche (Nutley, NJ). Hydroxylapatite (Bio Gel HTP) was obtained from Bio Rad (Richmond, CA). Triton X-100 and DTT (dithiothreitol) were purchased from Sigma Chemical Co. (St. Louis, MO). The butyl PBD scintillation cocktail was prepared from 5 g PBD (Beckman Instruments, Palo Alto, CA) in 1 L toluene (Mallinckrodt, St. Louis, MO). Tris-HCl was purchased from Calbiochem (Los Angeles, CA) and EDTA from Mallinckrodt (St. Louis, MO).

Results:

Competitive receptor studies of ^{3}H-24(R)25(OH)$_2D_3$ and ^{3}H-25(OH)D_3 in the cytosol of the chick parathyroid gland. Assuming that 24,25(OH)$_2D_3$ functions in a way similar to a hormone-like 1,25(OH)$_2D_3$, then it should have a specific binding protein/receptor in its target tissue. Therefore the 105,000 x g TED cytosol fraction obtained from a pool of 30 PTG from vitamin D-deficient cockerels was studied by in vitro incubation of ^{3}H-24,25(OH)$_2D_3$ ± 1000 fold unlabeled seco-steroid. The incubation was performed in TED buffer for 120 minutes at 4°C. The incubated cytosol was then placed on a 5-20% sucrose density gradient and centrifuged at 105,000 x g for 21.5 hours at 4°C. As

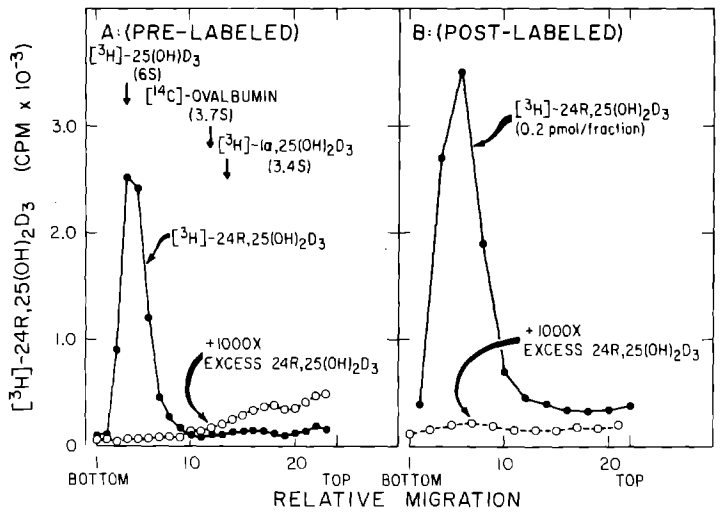

Figure 1: Sucrose gradient (5-20%) sedimentation of a low ionic strength (TED) 105,000 x g supernatant of chick parathyroid glands.

shown in Figure 1, it is apparent that in a low ionic strength (TED) 105,000 x g supernatant of the PTG, the $[^3H]$-24,25$(OH)_2D_3$ migrates in the 6S region of the gradient with no secondary peak in other regions. The 6S region of the gradient is also where the complex of the plasma D-binding protein and cytosol factor [see van Baelen et al. (15) and (16)] migrates with its ligand $[^3H]$-24,25$(OH)_2D_3$. The open circles in Figure 1 demonstrate that the $[^3H]$-24,25$(OH)_2D_3$ can be displaced from the 6 S region by an excess of 24,25$(OH)_2D_3$; similar results (not shown) were also obtained with nonradioactive 25$(OH)D_3$. Thus, this demonstrates that it would be difficult to differentiate between a $[^3H]$-24,25$(OH)_2D_3$ binding protein and the plasma-derived D-binding protein in a low ionic strength TED cytosol from chromatin. Similar results have been reported by Haussler et al. (17).

Elimination of the nonspecific $[^3H]$-24,25$(OH)_2D_3$ plasma-derived binding protein and detection of a specific binding protein for 24,25$(OH)_2D_3$:

As a consequence of our inability to detect the presence of a distinct binding protein for $[^3H]$-24,25$(OH)_2D_3$ in low ionic strength (TED) 105,000 x g supernatant fractions of PTG, alternative procedures were devised. Figure 2

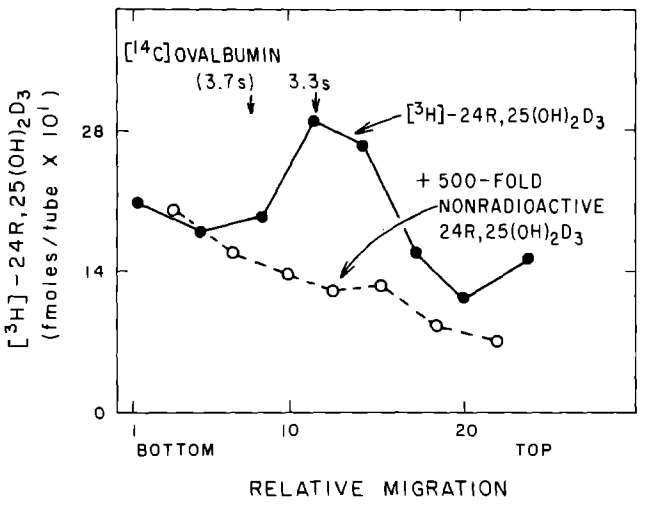

Figure 2: Sucrose gradient (5-20%) sedimentation of a high ionic strength extract of chromatin prepared in low ionic strength TED from chick parathyroid glands.

presents the results. A 20% full tissue PTG homogenate (30 glands) was incubated with [^{3}H]-24,25(OH)$_2$D$_3$ (1 pmole/1 ml homogenate) in TED for 120 min. at 4°C. Under these conditions receptor complexes (see below) were bound to the chromatin while some of the remaining [^{3}H]-24,25(OH)$_2$D$_3$ was also bound to the 6 S plasma binding protein. Next chromatin was prepared by washing and centrifugation with TED/0.5% Triton. This eliminated the free [^{3}H]-24,25-(OH)$_2$D$_3$ and the [^{3}H]-24,25(OH)$_2$D$_3$ bound to the 6 S plasma-derived protein. Next the chromatin pellet was incubated with 0.4 M KCl-TED and centrifuged (see Methods) and the resulting supernatant was placed on 5-20% sucrose high-salt density gradients. In order to overcome loss of the tritiated ligand from its binding protein/receptor during the long centrifugation, the separate collected fractions obtained after ultracentrifugation were re-incubated with [^{3}H]-24,25(OH)$_2$D$_3$ (0.2 pmole/fraction) $\pm$ 200 pmoles of nonradioactive 24,25(OH)$_2$D$_3$. The receptor-bound [^{3}H]-24,25(OH)$_2$D$_3$ was then detected by the HAP assay technique; as shown in Figure 2 a peak of specifically bound [^{3}H]-24,25(OH)$_2$D$_3$ was found in the gradient at a region which corresponds to the 3.3 S region of the gradient. There was no secondary peak in any other region and the peak was displaced by an excess of nonradioactive 24,25(OH)$_2$D$_3$.

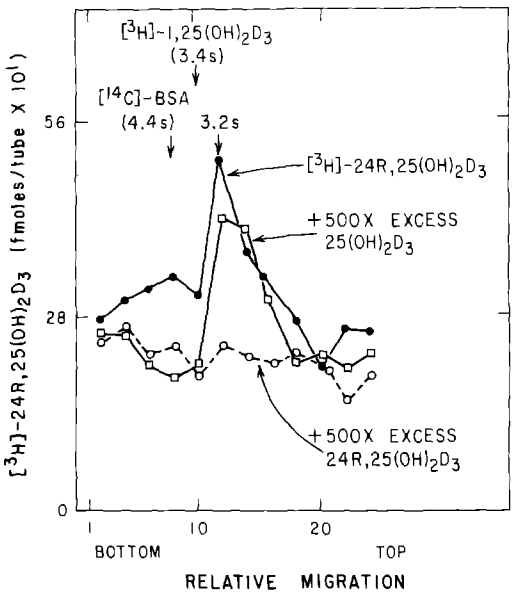

Figure 3: Sucrose gradient (5-20%) sedimentation of a chromatin extract from chick parathyroid gland with $[^3H]$-24,25$(OH)_2D_3$ and its competition by nonradioactive 24,25$(OH)_2D_3$ and 25$(OH)D_3$.

Clearly the plasma-derived 6 S protein was eliminated during the chromatin preparation. The parallel radioactivity present in only the prelabeled sucrose density gradient fractions and the successful refill of the receptors by "post labeling" suggests that $[^3H]$-24,25$(OH)_2D_3$ dissociates much more rapidly from this 3.2 S binding protein/receptor than does $[^3H]$-1,25$(OH)_2D_3$ from its 3.5-3.7 S PTG receptor (16,18).

Specificity of the PTG $[^3H]$-24,25$(OH)_2D_3$ receptor-like binding site against 25$(OH)D_3$: Several additional experiments were carried out to assess the specificity of the 3.2 S binding in the PTG chromatin extract. All gradient fractions were either postlabeled with 0.2 pmole $[^3H]$-24,25$(OH)_2D_3$ or competed with a 500 fold excess of nonradioactive 24,25$(OH)_2D_3$ or 25$(OH)D_3$ (see Figure 3). The $[^3H]$-24,25$(OH)_2D_3$ migrated again in the 3.2 S region. While the excess 24,25$(OH)_2D_3$ resulted in a full competition, an equivalent excess of 25$(OH)D_3$ did not; this demonstrates the specificity of the 3.2 S binding protein for 24,25$(OH)_2D_3$. It should be noted that the PTG $[^3H]$-1,25$(OH)_2D_3$

bound receptor migrates at an only slightly different position at 3.5-3.75 S (16,18); however no second "post labeling" incubation is required to demonstrate the presence of this receptor/binding protein.

Discussion: These results demonstrate for the first time the presence in a high ionic strength extract of chick parathyroid gland chromatin of a binding protein/receptor for $24,25(OH)_2D_3$. The relatively low concentration of the $24,25(OH)_2D_3$ binding protein and the observed requirement to "post label" the SDGA fractions emphasize the need for further studies to define optimal conditions for its stabilization. It also will be essential to study the relationship between the previously demonstrated PTG $1,25(OH)_2D_3$ receptor (16, 18) and the $24,25(OH)_2D_3$ receptor described in this report. Further studies, i.e. parallel post labeling with $[^3H]$-$25(OH)D_3$, $[^3H]$-$1,25(OH)_2D_3$, $[^3H]$-$24,25(OH)_2D_3$ $\pm$ nonradioactive metabolite, as well as Scatchard analysis studies of the different configurations (24R versus 24S) of $24,25(OH)_2D_3$, and determination of the "off-rate" of radioactive ligand all will provide the basis for a detailed comparison of biochemical properties. These data demonstrate that $24,25(OH)_2D_3$ has a biological activity and may act in a similar way (as a hormone) to the $1,25(OH)_2D_3$ metabolite with a specific receptor in the PTG. This would correspond to the physiological evidence of a role of $24,25(OH)_2D_3$ in bone mineralization, parathyroid hormone secretion and intestinal calcium absorption. It would support as well the studies performed on cultured chondrocytes which have shown that $24,25(OH)_2D_3$ increases the synthesis of sulfated protoglycans in human and rabbit growth plate chondrocytes, which are physiologically increased during growth periods (11,12).

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