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STUDIES ON THE MODE OF ACTION OF CALCIFEROL XVIII. EVIDENCE FOR A SPECIFIC HIGH AFFINITY BINDING PROTEIN FOR 1,25 DIHYDROXYVITAMIN D_3 IN CHICK KIDNEY AND PANCREAS[#]

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SUMMARY

Cytosol fractions prepared from rachitic chick kidney and pancreas were analyzed for binding of vitamin D_3 metabolites by sucrose density gradient centrifugation. Both cytosol fractions were found to contain a 3.6S macromolecule which specifically binds 1,25-dihdyroxy[^{3}H] vitamin D_3 and in addition a 5 to 6S macromolecule which binds 25-hydroxy[^{3}H]vitamin D_3. Sucrose gradient analysis of a KCl extract prepared from kidney or pancreas chromatin resulted in a peak (3.6S) of bound 1,25-dihydroxyvitamin D_3 which could not be distinguished from the cytoplasmic binding component. The interaction of 1,25-dihydroxy[^{3}H]vitamin D_3 with the cytoplasmic binding component of both tissues occurred at low concentrations of hormone with high affinity.

It has been found that vitamin D_3 which is first produced by the skin or taken in the diet, proceeds to the liver and is hydroxylated at the 25 position to yield 25-hydroxyvitamin D_3 (1,2). Next $25(OH)D_3$* is transported to the kidney where it is hydroxylated at the carbon 1 to yield 1,25-dihydroxyvitamin D_3. This seco steroid $1,25(OH)_2D_3$* is the most active metabolite of vitamin D_3 and is now considered to be a hormone (3). A current hypothesis concerning the mode of action of $1,25(OH)_2D_3$ in eliciting an increase in intestinal calcium transport is that the steroid associates first with a cytoplasmic protein receptor (4,5) in the target intestine. The cytoplasmic receptor plus $1,25(OH)_2D_3$ then move to the nucleus and saturate intestinal chromatin receptor binding sites (4-9) where it alters transcriptional events

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* Abbreviations employed are: $25(OH)D_3$ = 25-hydroxyvitamin D_3; $1,25(OH)_2D_3$ = 1,25-dihydroxyvitamin D_3.

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(10-12) and leads to the de novo synthesis of a vitamin D-dependent calcium binding protein (13,14).

That there is an interrelationship between pancreatic function and calcium metabolism has been shown in clinical studies in which both juvenile and adult onset diabetics are found to have significant bone disease (15,16). Also, Schneider et al. found that duodenal calcium absorption and levels of intestinal vitamin D dependent calcium binding protein (CaBP) are depressed in diabetic rats. Treatment with $1,25(OH)_2D_3$ but not $25(OH)D_3$ or vitamin D_3 restores duodenal calcium absorption and CaBP to normal (17,18). In the diabetic rat, the serum concentration is normal for $25(OH)D_3$ but is depressed to 1/8 normal for $1,25(OH)_2D_3$. Serum levels of $1,25(OH)_2D_3$ have been reported to be restored to control levels by insulin treatment (19). Finally, Christakos et al. (20) reported finding a 28,000 molecular weight protein in pancreas cytosol which is immunochemically undistinguishable from vitamin D dependent intestinal CaBP.

It has been reported that vitamin D acts at the kidney by affecting calcium and phosphate reabsorption (21,22). Also, vitamin D dependent calcium binding protein has been detected in chick, rat and human kidney (22-24). Chen and DeLuca (25) reported stimulation of [^{3}H]uridine incorporation into nuclear RNA of rat kidney by vitamin D metabolites. Christakos and Norman (26) recently reported that the vitamin D-dependent increase in kidney CaBP is accompanied by a parallel increase in the relative amount of mRNA coding for CaBP in the kidney, indicating that $1,25(OH)_2D_3$ may act as a steroid hormone not only in the intestine but also in the kidney. Thus due to the above observations suggesting actions of D-metabolites in both the kidney and pancreas it seemed appropriate to investigate the presence of specific receptors/binding proteins for vitamin D metabolites in these tissues.

Methods

White Leghorn cockerels (purchased from Pacesetter, Alta Loma, California) were raised for 4 weeks on a standard rachitogenic diet (27). 25 Hydroxy[^{3}H]-vitamin D_3 (9 Ci/mmol) and $1,25(OH)_2$[^{3}H]vitamin D_3 were obtained from Amersham Searle.

Chicks were sacrificed by decapitation; after exsanguination, the tissues were excised, cleaned of blood and rinsed in buffer (at 4°C) containing 0.3 M potassium chloride, 10 mM Tris-HCl, 1 mM EDTA, 0.5 mM dithiothreitol, pH 7.4. After washing, the organs were blotted, weighed and homogenized in 3 volumes (weight to volume) of the same buffer with a Potter Elvehjem homogenizer equipped with a Teflon pestle. Homogenates were centrifuged at 1,000 x g for 10 minutes and the resulting pellet was designated the crude nuclear fraction. The supernatant solution was then centrifuged at 100,000 x g for 1 hour in a Ti60 rotor in a Beckman L5-50 ultracentrifuge to yield a clear cytosol. Chromatin was prepared from the crude nuclear pellet by sequential homogenization and centrifugation at 4,300 x g for 10 minutes in 15 ml portions of 10 mM Tris-HCl, 0.5% Triton X-100 (pH 8.5) and 2 washes with 50 mM Tris-HCl (pH 7.5). Cytosol (200 µl) was incubated with 0.1 pmol $1,25(OH)_2[^3H]D_3$ (90-110 Ci/mmol) or 1 pmol $25(OH)[^3H]D_3$ for 90 minutes at 0°C. In experiments in which the chromatin bound receptor was investigated, aliquots (0.5 ml) of reconstituted chromatin cytosol preparations from 40% tissue homogenates prepared in 10 mM Tris-HCl, 1 mM EDTA, 0.5 mM dithiothreitol, pH 7.4 were incubated for 40 minutes at 23°C with 0.3 pmol of $[^3H]$-$1,25(OH)_2D_3$ (90-110 Ci/mmol). After incubation the chromatin was harvested, washed to remove nonspecifically bound steroid and extracted with 0.3 M KCl. Extracts were centrifuged at 100,000 x g for 30 minutes and the resulting supernatant solution was analyzed for steroid binding. Incubated cytosol and extracted chromatin were layered on 5-20% sucrose gradients prepared with a Buchler gradient mixer and a polystaltic pump in 0.3 M potassium chloride, 10 mM Tris-HCl, 1 mM EDTA, 12 mM thioglycerol, ph 7.4 and centrifuged at 0°C in a Beckman L5-65 ultracentrifuge using an SW 60 rotor at 50,000 rpm for 20-21 hours. ^{14}C ovalbumin, 3.67S, was used as a sedimentation marker and was run in a parallel gradient. Fractions (6 drops) were collected from the bottom and counted in 5 ml of aqueous scintillation cocktail.

Cytosol was assayed for specific binding of $1,25(OH)_2D_3$ by the hydroxylapatite binding assay. 0.2 ml of cytosol containing various concentrations of 1,25 dihydroxy$[^3H]$vitamin D_3 (9 Ci/mmol) were incubated for 18 hours at 0°C. Parallel incubations containing radioactive hormone plus 200-fold excess nonradioactive hormone were processed identically to yield values for nonspecific binding. Hydroxylapatite was used to separate bound from true ligand (28).

Results and Discussion

The binding *in vitro* of $1,25(OH)_2[^3H]D_3$ and $25(OH)[^3H]D_3$ at 0°C for 90 minutes to cytosol prepared from chick pancreas, kidney and duodenum was examined by sucrose density gradient centrifugation. As shown in Fig. 1 cytosols from chick pancreas and kidney contain one binding macromolecule for $1,25(OH)_2[^3H]D_3$ having a sedimentation coefficient of 3.6S and a separate binding protien for $25(OH)D_3$ with a sedimentation coefficient of 5-6S. An excess of nonradioactive $1,25(OH)_2D_3$ or $25(OH)D_3$ resulted in the disappearance of the respective 3.6 or 5.4S peak. These results are similar to those obtained when duodenal cytosol is incubated with $1,25(OH)_2[^3H]D_3$ or 25(OH)-

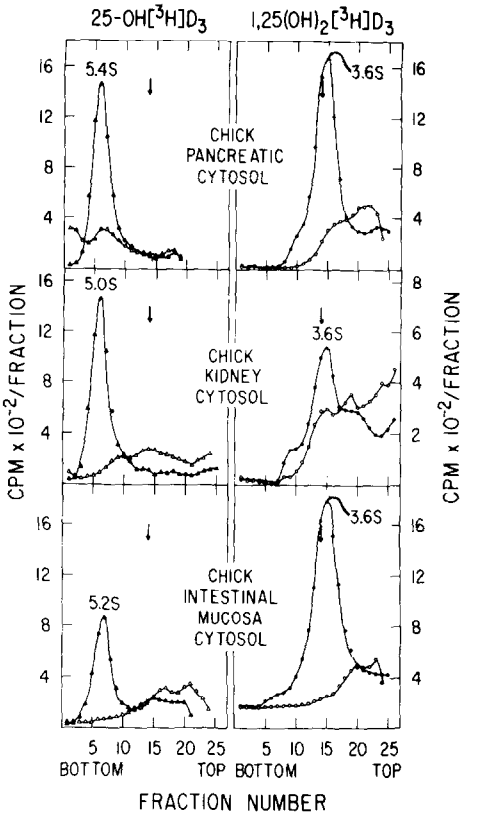

Fig. 1. Sucrose density gradient analysis of $1,25(OH)_2D_3$ and $25(OH)D_3$ binding components in cytosol from chick pancreas, kidney and intestine. ^{3}In the panels on the right, cytosols were prepared identically and incubated with 0.5 nM tritiated $1,25(OH)_2D_3$ (90-110 Ci/mmol) alone (●) or in the presence of 0.2 μM nonradioactive steroid (○) for 2 hr at 4°C. In the left panel separate and identical aliquots of cytosol were incubated with 4.5 nM $25(OH)[^3H]D_3$ alone (▲) or in the presence of 3.4 μM nonradioactive $25(OH)D_3$ (△) for 2 hr at 4°C. The arrow indicates the position of the [^{14}C]ovalbumin marker.

$[^3H]D_3$ and then analyzed by sucrose gradient centrifugation. Incubation of $1,25(OH)_2[^3H]D_3$ with cytosol from chick skeletal muscle, liver, or adrenal did not reveal specific hormone binding. When serum was incubated with [^{3}H] vitamin D metabolites for 90 minutes at 0°C and then applied to linear 5-20% sucrose density gradient and centrifuged the tritium peak migrates as a 4.1 S complex whether serum was incubated with $1,25(OH)_2[^3H]D_3$ or $25(OH)[^3H]$-D_3. Since there was no 4.1S peak in the sucrose density gradient analysis

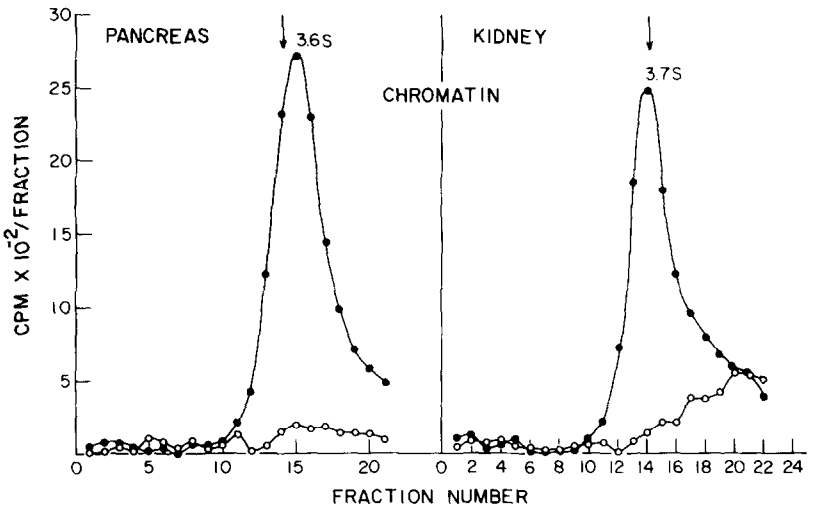

Fig. 2. The sucrose gradient sedimentation profile of the chromatin extractable $1,25(OH)_2D_3$ receptor from chick pancreas and kidney. Reconstituted cytosol and chromatin (0.5 ml) prepared from either chick pancreas or kidney was incubated with 1.4 nM [^{3}H]$1,25(OH)_2D_3$ (110 Ci/mmol) alone (●) or in the presence of 0.6 µM nonradioactive steroid (○) for 40 minutes at 23°C. The chromatin was harvested, washed and extracted with 0.3 M KCl. The extract was then sedimented through high salt 5-20% sucrose gradient. The arrow indicates the position of the [^{14}C]ovalbumin marker.

of the binding of tritiated vitamin D metabolites to kidney cytosol, the cytosol preparation was therefore not contaminated with serum.

After incubation *in vitro* of reconstituted cytosol and chromatin with $1,25(OH)_2[^3H]D_3$ at 24°C for 40 minutes, the KCl extractable material from the subsequently isolated chromatin fraction from chick pancreas or kidney was found to migrate as a 3.6S complex on a 5-20% sucrose density gradient (Fig. 2). Similar to the cytosol complex, binding to chromatin is specific since incubation in the presence of excess nonradioactive $1,25(OH)_2D_3$ results in the disappearance of the tritium peak.

The affinity and capacity of the kidney and pancreatic cytosol binding component for $1,25(OH)_2D_3$ was examined. Cytosol (0.2 ml) was incubated with increasing concentrations of $1,25(OH)_2[^3H]D_3$ and the specific binding was determined by the hydroxylapatite binding assay. As seen in Fig. 3 the binding proteins in both kidney and pancreatic cytosol have a low capacity for

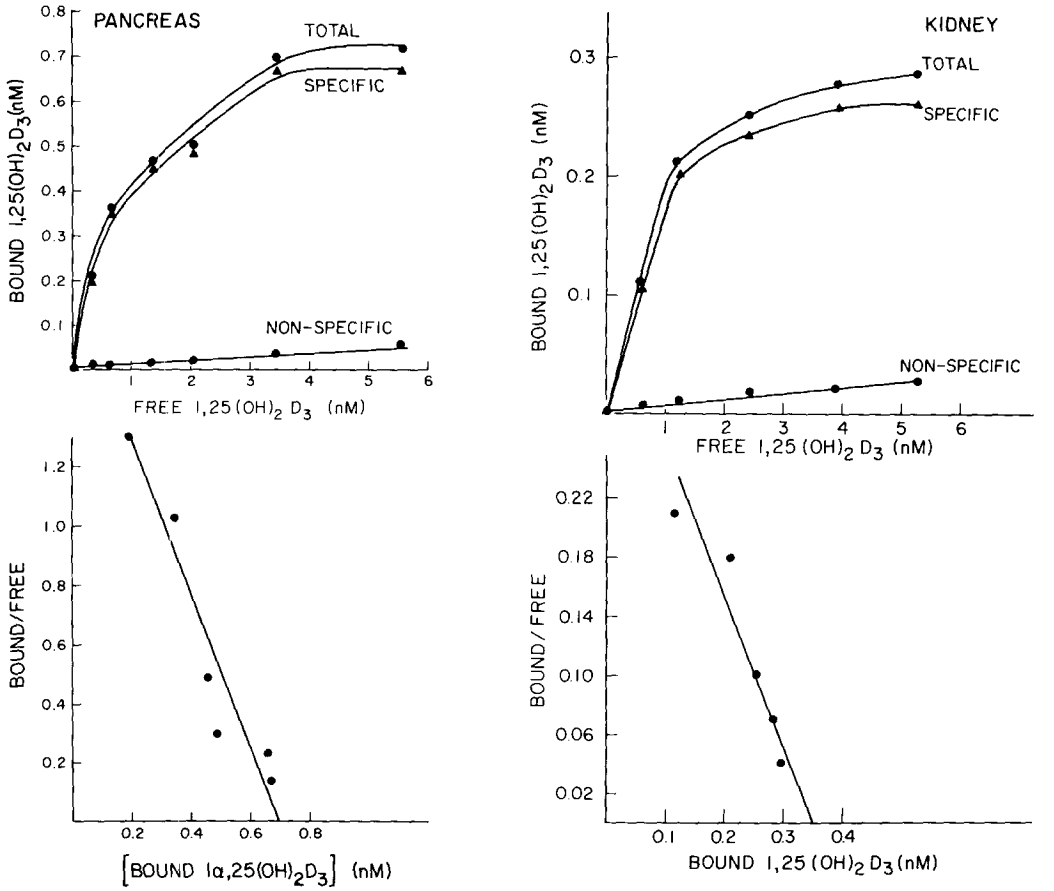

Fig 3. Scatchard plots of the binding of $1,25(OH)_2D_3$ to chick pancreas and kidney cytosol receptors. Aliquots of cytosol (200 µl and 4 mg protein for chick pancreas; 200 µl and 6 mg protein for chick kidney) were incubated with increasing amounts of $1,25(OH)_2[^3H]D_3$ in the presence (nonspecific binding) or absence (total binding) of nonradioactive ligand at 0°C for 18 hours. Bound and free steroid were separated by hydroxylapatite. The bottom panel of the figures gave K_d values of 4.1×10^{-10} M for the binding of $1,25(OH)_2D_3$ to pancreas cytosol and 1.2×10^{-9} M for the binding of $1,25(OH)_2D_3$ to kidney cytosol.

steroid with a small amount of nonspecific binding. A Scatchard analysis of the specific cytosol binding is shown in Fig. 3. The equilibrium dissociation constant for the hormone macromolecular complex at 0°C for pancreas was 4.2×10^{-10} M and for kidney it was 1.2×10^{-9} M as determined by the hydroxylapatite procedure.

The data presented in this paper demonstrate a specific high-affinity, low-capacity binding protein for $1,25(OH)_2D_3$ in chick kidney and pancreas

cytosol as well as specific binding of $1,25(OH)_2D_3$ to chromatin in these tissues. These observations strongly suggest that $1,25(OH)_2D_3$ acts as a steroid hormone not only in intestine and parathyroid glands but also in chick kidney and pancreas. Wecksler et al. (29-30) have compared the biochemical properties for the $1,25(OH)_2D_3$ cytosol binding protein/receptor in chick intestine and parathyroid gland, rat intestine, and human intestine and parathyroid gland adenoma. All were found to migrate at 3.6 to 3.7S on 5-20% sucrose gradients and all had molecular weights in the range of 60-70,000 with a K_D of 1.8-5.4 x 10^{-10} M. Thus there seems to be a high degree of homology in certain physical properties of the cytosol receptors for $1,25(OH)_2D_3$ present in its target tissues. Work is currently in progress to further biochemically characterize these two new binding protein/receptors for $1,25(OH)_2D_3$.

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