NUCLEAR AND CYTOPLASMIC RECEPTORS FOR 1,25-DIHYDROXYCHOLECALCIFEROL IN INTESTINAL MUCOSA[+]

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Summary: The apparent hormonal form of cholecalciferol, 1,25-dihydroxycholecalciferol (1,25-$(OH)_2$-CC), was incubated with intestinal mucosa homogenates and whole intestinal tissue, in vitro. After 40-70 min, 1,25-$(OH)_2$-CC was specifically associated with the nuclear chromatin fraction. This sterol remains bound to the cytosol fraction at 0°C and a dramatic movement to the nuclear chromatin occurs at 37°C indicating that the subcellular localization of the sterol is temperature dependent. Isolated intestinal cytosol, previously incubated with 1,25-$(OH)_2$-CC, is required for transportation of the hormone to the intestinal chromatin fraction; cytosol fractions from other tissues are ineffective mediators of this sterol migration. It is concluded that the intestinal cytosol contains a specific receptor that functions to transport 1,25-$(OH)_2$-CC to the nucleus, its probable site of action.

Cholecalciferol (vitamin D_3) is converted to 25-hydroxycholecalciferol (25-(OH)-CC)* in several tissues, including the liver (1,2), and this metabolite is converted to 1,25-dihydroxycholecalciferol (1,25-$(OH)_2$-CC) in the kidney (3). After injection of radioactive cholecalciferol into a vitamin D-deficient animal, 1,25-$(OH)_2$-CC is the major form of the sterol found in the intestine (4). This metabolite is the most potent and fastest acting form of the vitamin in stimulating calcium transport across the intestine (5). 1,25-$(OH)_2$-CC is apparently the metabolite of vitamin D_3 that mediates the biochemical events that result in increased calcium absorption from the intestine and is considered to be the hormonal form of the vitamin (6-10). Previous studies of the subcellular distribution of cholecalciferol and its metabolites following injection of radioactive cholecalciferol (4) or 25-(OH)-CC and 1,25-$(OH)_2$-CC (11) into rachitic animals have shown that 1,25-$(OH)_2$-CC is localized in the nuclear chromatin fraction of intestinal mucosa. Further investigation of the chromatin-

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*Abbreviations used: 25-OH-CC, 25-hydroxycholecalciferol; 1,25-$(OH)_2$-CC, 1,25-dihydroxycholecalciferol; sucrose-Tris-K-Mg, 0.25 M Sucrose in 0.05 M Tris-HCl, pH 7.4, 0.025 M KCl, 0.005 M $MgCl_2$.

associated binding site indicated that 1,25-dihydroxycholecalciferol is non-covalently linked with a chromosomal protein that is not a histone (12).

The present communication describes further elucidation of the specific binding of 1,25-$(OH)_2$-CC to the chromatin fraction of intestinal mucosa. Direct addition of the sterol to tissue homogenates, *in vitro*, shows that 1,25-$(OH)_2$-CC binds effectively only to chromatin isolated from a target tissue such as the intestine. Other sterols are not associated with intestinal chromatin under identical incubation conditions. We also present evidence for the existence of a cytoplasmic receptor that is required to transfer 1,25-$(OH)_2$-CC to the mucosal cell nucleus.

MATERIALS AND METHODS

Materials. Animals used in all experiments were White Leghorn cockerels that were raised for 3-4 weeks on a vitamin D-deficient diet (13). 25-Hydroxy[26(27)-methyl-$^3H_2$]cholecalciferol (19.7 Ci/mmole) and [α1,2-$^3H_2$]cholecalciferol (0.577 Ci/mmole) were obtained from Amersham/Searle. Nonradioactive cholecalciferol was purchased from Calbiochem; crystalline 25-hydroxycholecalciferol was a gift from Dr. John C. Babcock of the Upjohn Co. [6,7-$^3H_2$]Estradiol-17β (46.6 Ci/mmole) was obtained from New England Nuclear Corp. and nonradioactive estradiol-17β was obtained from Sigma Chemical Co.

Preparation of 1,25-Dihydroxy[26(27)-Methyl-$^3H_2$]cholecalciferol. [^3H]1,25-$(OH)_2$-CC was prepared from [^3H]25-OH-CC by a modification (10) of the procedure of Lawson *et al.* (7). Purification of the generated [^3H]1,25-$(OH)_2$-CC was carried out via chromatography on columns of Sephadex LH-20 and Celite as described elsewhere (9,10). The resulting [^3H]1,25-$(OH)_2$-CC was diluted to a specific activity of 1000 dpm/pmole.

Incubations. Small intestinal mucosa and liver homogenates were prepared by homogenizing 3 gms of tissue in 30 ml of 0.25 M sucrose in 0.05 M Tris-HCl, pH 7.4, 0.025 M KCl, and 0.005 M $MgCl_2$ (sucrose-Tris-K-Mg). Homogenization was performed with a Potter-Elvehjem homogenizer equipped with a Teflon pestle. The resulting homogenates were incubated with various sterols for 70 min at

25°C. Whole small intestines were slit endwise to expose the mucosal surface and were incubated at 37° or 0° for various periods of time under an atmosphere of 95% O_2-5% CO_2 in 15 ml of Eagle's medium. All sterols were added to the incubation medium in 200 µl ethanol.

Preparation of Subcellular Fractions. Subcellular fractions were prepared by the method of Haussler et al. (4). Chromatin was washed three times with 1% Triton X-100 in 0.01 M Tris, pH 7.5.

Radioactivity Measurement. The sterols of the subcellular fraction of interest were extracted by modification (4) of the method of Bligh and Dyer (14) and radioactivity was determined as previously described (4).

DNA Assay. DNA was determined by the diphenylamine method of Dische (15).

RESULTS AND DISCUSSION

In initial experiments, 130 pmoles of [^{3}H]1,25-$(OH)_2$-CC were incubated directly with intestinal homogenates for 70 minutes at 25°C.[1] Radioactivity was found in the mitochondrial (20%), microsomal (13%), cytosol (22%), and crude nuclear fractions (45%). Only the chromatin fraction prepared from the crude nuclei exhibited the characteristic of saturability, an accepted property of receptor systems (16). Thus, while radioactivity increased linearly with increasing amount of added 1,25-$(OH)_2$-CC in most subcellular fractions, the chromatin contained a finite number of binding sites for the hormone. Figure 1 shows the amount of chromatin associated radioactivity as a function of the amount of 1,25-dihydroxy[^{3}H]cholecalciferol in the medium. Extraction of sterols from the chromatin fraction and chromatography on Sephadex LH-20 and Celite liquid-liquid partition columns showed that the radioactive sterol bound to the chromatin was unmetabolized [^{3}H]1,25-$(OH)_2$-CC. The amount of [^{3}H]1,25-$(OH)_2$-CC bound at saturation corresponds to 14 pmoles per intestine (15 mg DNA equivalent), a value that is two to three times greater than reported in previous studies of this receptor, in vivo (11, 17). This higher saturation level sug-

[1] Homogenates consisted of approximately 1.5 g of tissue in 17 ml.

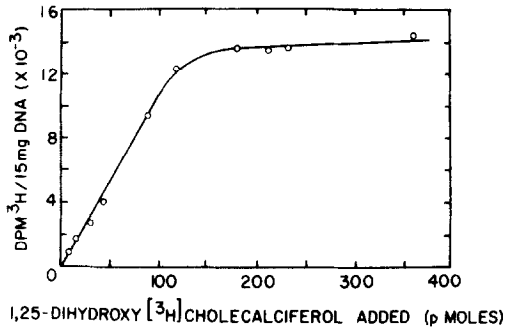

Fig. 1.

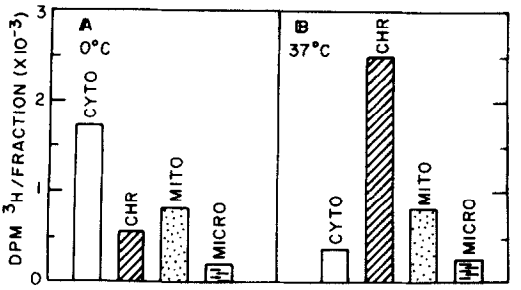

Fig. 2.

Fig. 1. Saturation of radioactivity in chromatin after incubation of intestinal homogenate with [^{3}H]1,25-$(OH)_2$-CC. Homogenates (17ml) of intestinal mucosa from vitamin D-deficient chicks were incubated with increasing amounts of [^{3}H]1,25-$(OH)_2$-CC for 70 min at 25°C. It was found in preliminary experiments that intestines of 3-4 week old rachitic chicks routinely yield approximately 3 gm (wet weight) of mucosa. Three grams of mucosa contain 15 mg of DNA and, to eliminate variations in tissue size, all results were corrected to that value based upon individual DNA determinations. The results presented represent a composite of three separate experiments.

Fig. 2. Temperature dependent movement of 1,25-$(OH)_2$-CC from cytosol to chromatin fraction. Abbreviations used for subcellular fractions are: CYTO, cytosol; CHR, chromatin; MITO, mitochondria; MICRO, microsomes. Whole intestines were first incubated with 1,25-$(OH)_2$-CC (65 pmoles) at 0° for 15 min, washed with sucrose-Tris-K-Mg, transferred to a sterol free Eagle's medium and further incubated as follows: A) 40 min at 0°C and B) 40 min at 37°C. Sterols were extracted from the fraction of interest and tritiated sterols counted as described in Methods. Each fraction is corrected to 15 mg DNA or 3 gm of tissue equivalent (see legend to Fig 1). At 37°C, approximately 25% of the radioactivity present in the crude nuclear fraction was associated with the final purified chromatin.

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gests that more receptors are exposed to the sterol in the homogenate than in the intestine, in vivo.

In order to test the selectivity of this receptor fraction, closely related sterols were incubated with mucosa homogenates. The data in Table I indicate that 1,25-$(OH)_2$-CC is bound to the chromatin fraction far more effectively than the other sterols tested. In addition, 1,25-$(OH)_2$-CC binds more efficiently to the intestinal chromatin than to the chromatin from a non-target organ, the liver. This evidence suggests that the chromatin receptor for 1,25-$(OH)_2$-CC is specific for this sterol and occurs only in the target organ.

It is known that steroid hormones such as estradiol-17β (18) and progesterone

Table I. Sterol and tissue specificity of the localization of 1,25-$(OH)_2$-CC in the chromatin and cytosol fractions of intestinal mucosa homogenates. Homogenates (17 ml) of intestines of vitamin D-deficient chicks were incubated with 65 pmoles of [^{3}H]1,25-$(OH)_2$-CC, [^{3}H]25-OH-CC, [^{3}H]cholecalciferol, or [^{3}H]estradiol-17β for 70 min at 25°C. A liver homogenate (17 ml) was incubated with [^{3}H]1,25-$(OH)_2$-CC under the same conditions to determine tissue specificity of binding.

Sterol Added	Tissue	Localization of Radioactivity[a]	
		Chromatin ^{3}H DPM/15 mg DNA	Cytosol ^{3}H DPM/Fraction[b]
[^{3}H]1,25-$(OH)_2$-CC	Intestine	7,200	23,000
[^{3}H]25-OH-CC	Intestine	200	20,000
[^{3}H]cholecalciferol	Intestine	150	5,300
[^{3}H]estradiol-17β	Intestine	300	4,400
[^{3}H]1,25-$(OH)_2$-CC	Liver	350	25,000

[a]All radioactive sterols diluted with the corresponding unlabeled compounds to yield a working specific activity of 1000 dpm/pmole.
[b]Cytosol equivalent from 3 gms of tissue.

(19), which have chromatin-associated receptors, are transferred to the nucleus in their respective target organs by a cytoplasmic receptor. Following incubation of 1,25-$(OH)_2$-CC with intestinal mucosa homogenates this sterol was present in the cytosol fraction (Table I). However, the specificity of this fraction was rather low, for 25-(OH)-CC also associates with the intestinal cytosol fraction and 1,25-$(OH)_2$-CC can bind effectively to liver cytosol. Thus, based strictly upon binding to the cytosol fraction, these data do not demonstrate that a specific cytosol receptor for 1,25-$(OH)_2$-CC exists in intestine.

To examine further the intestinal cytosol fraction and its possible role in transferring 1,25-$(OH)_2$-CC to the nuclear binding sites, 1,25-dihydroxy[^{3}H]-cholecalciferol was added to whole intestinal tissue in Eagle's medium. Whole intestines were first incubated for 15 min at 0°C with [^{3}H]1,25-$(OH)_2$-CC. The intestines were then rinsed, transferred to a medium containing no 1,25-$(OH)_2$-CC, and further incubated for 40 min at either 0°C or 37°C. Localization of radioactivity in the cytosol fraction occurs when the tissue is incubated with 1,25-$(OH)_2$-CC at 0°C (Fig 2A). However, if the tissue is incubated at 37°C

Table II. Transfer of 1,25-$(OH)_2$-CC from cytosol fraction of several tissues to the nucleus of the intestinal mucosa. Cytosol fractions (15 ml) of the liver, kidney and intestine were prepared as described in Methods and incubated for 15 min at 25° with 13 pmoles [^{3}H]1,25-$(OH)_2$-CC. These fractions then were mixed with sucrose-Tris-K-Mg-washed nuclear fractions of intestinal mucosa and incubated at 25°C for 60 min.

Cytosol incubated with [^{3}H]1,25-$(OH)_2$-CC	Radioactivity Detected in Mucosa Chromatin (^{3}H DPM/15 mg DNA)
Intestine	8300
None[a]	370
Intestine (boiled)[b]	460
Liver	1000
Kidney	670

[a] 13 pmoles [^{3}H]1,25-$(OH)_2$-CC were incubated with nuclei suspended in 15 ml of sucrose-Tris-K-Mg.

[b] Intestinal cytosol was heated for 15 minutes at 100°C and then cooled to 25°C before addition of 1,25-$(OH)_2$-CC.

after an initial period at 0°C, the association of the hormone with the cytosol fraction is reduced, and the 1,25-$(OH)_2$-CC is found primarily in the chromatin fraction (Fig 2B). The results in Fig 2 suggest that there was a temperature dependent movement of 1,25-$(OH)_2$-CC from the cytosol to the nucleus.

In order to demonstrate that 1,25-$(OH)_2$-CC is actually being transported from cytosol to nuclear chromatin, intestinal cytosol was isolated and incubated with [^{3}H]1,25-$(OH)_2$-CC. This cytosol fraction, containing bound [^{3}H]1,25-$(OH)_2$-CC, was then incubated at 25°C with isolated intestinal nuclei. Table II shows that the chromatin isolated from these nuclei contained [^{3}H]1,25-$(OH)_2$-CC. The data in Table II also demonstrate that the intestinal cytosol fraction is an obligatory requirement for transfer of the hormone. 1,25-$(OH)_2$-CC was not transported to chromatin by adding the sterol, suspended in sucrose-Tris-K-Mg, to isolated nuclei. Furthermore, boiling the intestinal cytosol destroys its ability to transfer 1,25-$(OH)_2$-CC to the nucleus. The fact that boiled cytosol or sucrose-Tris-K-Mg cannot mediate the transfer of sterol to the nucleus indicates that the sterol must first bind to a thermolabile component of intestinal cytosol.

Cytosol fractions from other tissues are not effective mediators of this transfer (Table II). Thus, it is concluded that there is a component unique to the intestinal cytosol that is necessary for the transfer of 1,25-$(OH)_2$-CC to the intestinal nucleus. The thermal lability of this cytosol component suggests that it is a protein receptor for 1,25-$(OH)_2$-CC.

Additional characterization of the proposed receptor molecules for 1,25-$(OH)_2$-CC in cytosol and chromatin is required before conclusions can be drawn as to their functional interrelationship and their role in the molecular mode of action of the hormone. It is possible that 1,25-$(OH)_2$-CC acts analogously to other steroid hormones to regulate gene expression (16, 18-20).

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