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Mechanism of Action of 1,25-Dihydroxycholecalciferol on Intestinal Calcium Transport

(rat/vitamin D/actinomycin D/kidney/"peak V" metabolite)

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ABSTRACT The prior administration of actinomycin D prevents the metabolism of [^{3}H]25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol, a metabolite of vitamin D_3 that is effective in the stimulation of intestinal calcium transport. In this paper, the question of whether the response of intestinal calcium transport to 1,25-dihydroxycholecalciferol is sensitive to actinomycin D was examined. While the response of intestinal transport to physiological amounts of 25-hydroxycholecalciferol is blocked by actinomycin D, the response of intestinal calcium transport to 1,25-dihydroxycholecalciferol is insensitive to the antibiotic. These results suggest that 1,25-dihydroxycholecalciferol, or a further metabolite thereof, is the metabolically active form of vitamin D in the intestine, that it functions by a process not involving transcription of DNA, and that the step sensitive to actinomycin D in the action of vitamin D on the intestine does not occur in the intestine, but is the conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol in the kidney.

In 1965, Zull *et al.* (1) conclusively demonstrated that actinomycin D given prior to, but not after, vitamin D prevents completely the action of the vitamin in increasing intestinal calcium transport and bone mineral mobilization. At the same time, Norman (2) reported that actinomycin D blocks vitamin D-stimulated intestinal absorption of calcium in chickens. Further studies (3) provided very strong evidence that the mechanism of vitamin D action both in the intestine and in bone must involve transcription of DNA and protein synthesis. The concept was further strengthened when it was demonstrated that nuclear RNA labeling by [^{3}H]orotic acid or [^{3}H]uridine could be stimulated by vitamin D in both rats (4) and chicks (5). Since that time, it has been assumed that vitamin D must initiate these actions by activating a gene(s) in the intestine that in turn provides the messenger RNA for the synthesis of protein components required for calcium transport or bone mineral mobilization.

Lund and DeLuca (6), following still another lead with regard to the mechanism of vitamin D action, were able to demonstrate quite convincingly the existence of a metabolite(s) of vitamin D more active than the parent vitamin itself. Furthermore, this metabolite acted more rapidly in initiating intestinal calcium transport than did vitamin D_3 itself (7), leading to the concept of metabolically active forms of vitamin D. Subsequently, this metabolite was isolated in pure form from the plasma of pigs and was identified as 25-hydroxycholecalciferol 25-(OH)D_3 (8). Strong evidence has since then been provided that 25-hydroxylation represents the initial step in the functional metabolism of the vitamin (9). Still other work has demonstrated that 25-(OH)D_3 represents the major circulating active form of the vitamin and thus may be considered a hormonal form (9, 10). With radioactive 25-(OH)D_3, Cousins *et al.* (11, 12) demonstrated the accumulation of a metabolite more polar than 25-(OH)D_3 in intestine and bone, as well as other tissues, that could well represent a metabolically active form of vitamin D (10, 13, 14). Haussler *et al.* (13), and more recently Myrtle *et al.* (15) and Kodicek *et al.* (16), have provided evidence that this metabolite has potent biological activity in stimulating intestinal calcium transport. This metabolite acts more rapidly in initiating intestinal calcium transport than does 25-(OH)D_3 (17–19), but it is less effective in curing rickets in rats than 25-(OH)D_3 (18) and is no more active than 25-(OH)D_3 in stimulating bone mineral mobilization (18). In any case, the present evidence would strongly suggest that this polar metabolite ("peak V") represents the metabolically active form of the vitamin in the intestine. This metabolite was recently isolated in pure form from intestine and identified conclusively as 1,25-$(OH)_2D_3$ (20), in agreement with a structure deduced (21) from a preparation of 30% purity.

Of significance is the recent finding (22) that actinomycin D or cycloheximide given prior to the injection of radioactively labeled 25-(OH)D_3 prevents the metabolism of 25-(OH)D_3 to 1,25-$(OH)_2D_3$; the 25-(OH)D_3 then accumulates in the intestine. When actinomycin D or cycloheximide is given after 25-(OH)D_3, these agents are no longer able to block its metabolism to 1,25-$(OH)_2$ D_3. These results suggest that 25-(OH)D_3 itself induces the formation of an enzyme or enzymes that are responsible for the production of 1,25-$(OH)_2$ D_3. It is possible that the actinomycin D-sensitive step in the metabolic function of vitamin D is the conversion of 25-(OH)D_3 to the polar metabolite, which in turn functions in the intestine to initiate intestinal calcium transport.

This report provides unequivocal proof that the 1,25-$(OH)_2$ D_3, or a further metabolite thereof, represents the metabolically active form of the vitamin in the intestine. Furthermore, we show that this metabolite initiates intestinal calcium transport by a process that does not involve transcription of DNA into RNA; thus, the actinomycin D-sensitive step in vitamin D function in the intestine is the conversion of 25-(OH)D_3 to 1,25-$(OH)_2D_3$.

Abbreviations: 25-(OH)D_3, 25-hydroxycholecalciferol; 1,25-$(OH)_2D_3$, 1,25-dihydroxycholecalciferol.

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MATERIALS AND METHODS

Young male weanling rats were obtained from the Holtzman Co., Madison, Wis. They were housed individually in hanging wire cages and fed an adequate calcium (0.47%) and phosphorus (0.3%) diet deficient in vitamin D (23). For all of the intestinal calcium transport work, the rats were fed the adequate calcium and phosphorus diet for 4–5 weeks, at which time they ceased to grow and exhibited a severe hypocalcemia (about 4–5 mg calcium/100 ml of plasma); these rats were used for the everted intestinal sac experiments.

Radioactive Compounds. The [1,2-^{3}H]vitamin D_3 was synthesized as previously described (24), and had a specific activity of 0.6 Ci/mmol. The [26,27-^{3}H]25-(OH)D_3 had a specific activity of 1.3 Ci/mmol (25).

Preparation of 1,25-(OH)$_2D_3$. 25-(OH)D_3 was kindly supplied in crystalline form by the Philips-Duphar Co. of The Netherlands. 1,25-(OH)$_2D_3$ was isolated from intestinal mucosa of chicks given [1,2-^{3}H]vitamin D_3 (18, 20). Vitamin D_3 is a commerical product purchased from the Philips-Roxane Co. of the United States.

Intestinal calcium transport measurements

The vitamin D-deficient rats were maintained on the 0.47% calcium, 0.3% phosphorus diet for 5 weeks and divided into an appropriate number of groups. Where indicated, 1 μg of actinomycin D per g of body weight was injected intrajugularly 2 hr before the animals were injected with the appropriate metabolite in the jugular vein. Control animals received the vehicle alone at the appropriate times. The animals were killed either 8 or 12 hr later. Intestinal calcium transport was measured by the everted sac technique (26).

Metabolism experiments with [26,27-^{3}H]25-(OH)D_3

Rats fed the appropriate diet were given actinomycin D (Nutritional Biochemicals, Inc., Cleveland, Ohio) (1 μg/g body weight) intrajugularly 2 hr before they received 65 pmol of [^{3}H]25-(OH)D_3. The control animals received the appropriate volume of vehicle at the indicated times. The rats were killed by decapitation and the intestine or kidneys were immediately excised. The intestine was slit lengthwise and rinsed in ice-cold isotonic saline, and the mucosa were scraped from the intestine, which rested on a stainless-steel plate embedded in ice. A tissue homogenate (25% in distilled water) was prepared and then immediately extracted with methanol and chloroform (6). The chloroform extracts thus obtained were chromatographed on 2 × 20 cm Sephadex LH-20 gel columns in a solvent of 65% chloroform in petroleum ether (bp 67–69°C) (27) for the resolution of polar metabolites of vitamin D. Fractions were collected from the chromatographic columns and dried directly under a stream of air in liquid-scintillation counting vials. To each vial was added 15 ml of a toluene-based counting solution (23), and the tritium content was determined by means of a Packard liquid scintillation counter, model 3375, equipped with external standardization for the determination of disintegrations per minute.

RESULTS

We had previously demonstrated that accumulation in the intestine of 1,25-(OH)$_2D_3$ reached a plateau about 8 hr after injection of [^{3}H]25-(OH)D_3 to vitamin D-deficient rats (11, 12, 22). It is evident from the present experiments that 8 hr after injection of vitamin D-deficient rats with [^{3}H]25(OH)D_3 there is a marked accumulation of the 1,25-(OH)$_2$ D_3 in the intestinal mucosa. In agreement with previous results, the prior administration of actinomycin D to these vitamin D-deficient rats markedly inhibited the accumulation of 1,25-(OH)$_2D_3$ in the intestine (Fig. 1). Because it appears that the kidney is the primary, if not the sole, site of synthesis of 1,25-(OH)$_2D_3$ (28, 29), it was of interest to determine whether inhibition of the accumulation of 1,25-(OH)$_2D_3$ could be observed in this tissue. Clearly, the actinomycin D also prevented the appearance of 1,25-(OH)$_2D_3$ in the kidney after injection of 25-(OH)D_3 (Fig. 1). These findings, and those suggesting that 1,25-(OH)$_2$ D_3 represents the metabolically active form of 25-(OH)D_3 in the intestine, raise the question of whether the actinomycin D-sensitive step in the vitamin D-induced increase in intestinal calcium transport might not be the formation of 1,25-(OH)$_2$ D_3 in the kidney.

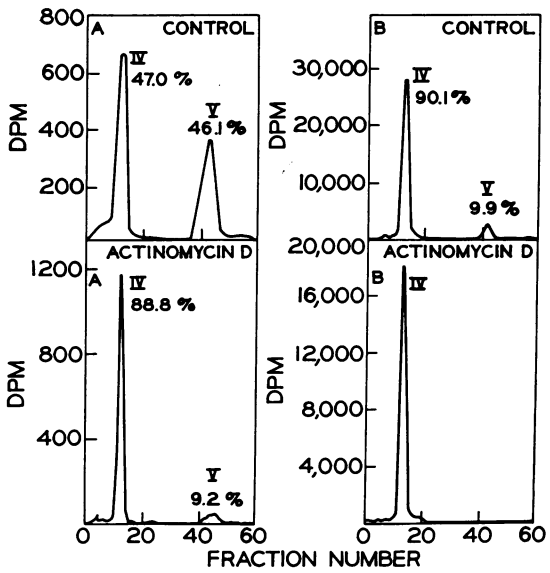

FIG. 1. Actinomycin D inhibition of the metabolism of 25-(OH)D_3 to 1,25-(OH)$_2D_3$. Vitamin D-deficient rats were given 1 μg/g body weight of actinomycin D 2 hr prior to a 65-pmol dose of [^{3}H]25-(OH)D_3 (0.05 ml, i.v., 95% ethanol). Controls received ethanol only. All rats were killed 8 hr after the administration of [^{3}H]25-(OH)D_3. Chloroform extracts were prepared of (*A*) intestinal mucosa and (*B*) kidney. Chromatography was on a LH-20 column (2 × 20 cm) with a solvent of 65% chloroform in Skellysolve B (bp 67–69°C) (26). Peak IV = unchanged 25-(OH)D_3, while peak V = biologically active 1,25-(OH)$_2$ D_3 (18). The figures near the peaks represent the % of chromatographed radioactivity.

The results shown in Table 1 provide the necessary evidence that the action of 25-(OH)D_3, like that of vitamin D (1, 3), in inducing intestinal calcium transport is blocked by the prior administration of actinomycin D, although at doses higher than 65 pmol of 25-(OH)D_3 per rat the block is incomplete. This may be related to the incomplete block in the production of 1,25-(OH)$_2D_3$ by actinomycin (Fig. 1) (22). These results appeared to be consistent with the previous data concerning the block of vitamin D action by actinomycin D, since 65 pmol of 25-(OH)D_3 can be considered to be a physiological dose. Of central importance is the finding that the rise in intestinal calcium transport that is induced by 65 pmol of

TABLE 1. *Failure of actinomycin D to inhibit the rise in intestinal calcium transport induced by 1,25-(OH)$_2$D$_3$*

Treatment		Transport
Actinomycin D	Vitamin D metabolite	^{45}Ca inside/^{44}Ca outside
−	—	1.7 ± 0.2*
−	25-(OH)D$_3$	2.7 ± 0.1†
+	25-(OH)D$_3$	1.8 ± 0.2
−	1,25-(OH)$_2$D$_3$	2.9 ± 0.2†
+	1,25-(OH)$_2$D$_3$	3.1 ± 0.3†

Rats were fed the 0.47% calcium, 0.3% phosphorus diet deficient in vitamin D for 5 weeks. Where indicated, each rat received 1 μg/g body weight of actinomycin D intrajugularly in 0.05 ml of ethanol. 2 hr later, where indicated, each rat received 65 pmol of 25-(OH)D$_3$ or 1,25-(OH)$_2$D$_3$ intrajugularly in 0.05 ml of ethanol. Control rats received the appropriate amount of ethanol in each case at the appropriate time. All rats were killed 12 hr after 25-(OH)D$_3$ or 1,25-(OH)$_2$D$_3$ administration. Intestinal calcium transport was measured as described by Martin and DeLuca (25).

* Standard deviation. There were four rats in each group.

† Significantly different from control group ($P < 0.01$ by Student's *t*-test).

1,25-(OH)$_2$D$_3$ is insensitive to actinomycin D (Table 1). These results are of fundamental importance in two regards. One, they provide firm evidence that 1,25-(OH)$_2$D$_3$, or a further metabolite thereof, must be the metabolically active form of the vitamin in the intestine. Even more important, they provide evidence that the intestinal calcium transport initiated by 1,25-(OH)$_2$D$_3$ does not involve transcription of DNA into RNA.

DISCUSSION

Our results demonstrate that 1,25-(OH)$_2$ D$_3$, or a further metabolite thereof, must be the metabolically active form of vitamin D$_3$ in the intestine. This conclusion is based entirely on the fact that in the presence of actinomycin D, 25-(OH)D$_3$ does not act, whereas the 1,25-(OH)$_2$D$_3$ is able to initiate intestinal calcium transport. This finding, taken together with the fact that actinomycin D prevents the metabolism of 25-(OH)D$_3$ to 1,25-(OH)$_2$D$_3$, can only suggest that 25-(OH)D$_3$ is the precursor of the metabolically active form of vitamin D in the intestine. The results presented here also provide a new insight into the mechanism of vitamin D action in the intestine, namely that 1,25-(OH)$_2$D$_3$ can initiate intestinal calcium transport in the presence of actinomycin D. This finding suggests that transcription of DNA into RNA is not involved in vitamin D$_3$ action once the correct, metabolically active, form of the vitamin is provided.

Whether protein synthesis is required for the action of vitamin D$_3$ cannot be deduced from the present experiments. Our attempts to use cycloheximide to examine this question have not as yet proven fruitful. The results in the present report also support the previous conclusion that 25-(OH)D$_3$ probably induces the formation of an enzyme or enzymes involved in the production of 1,25-(OH)$_2$D$_3$. Recently, it has been clearly demonstrated (28, 29) that the kidney is the primary, if not the sole, site of synthesis of 1,25-(OH)$_2$D$_3$. This finding raises the question of why tritium derived either from vitamin D$_3$ or from 25-(OH)D$_3$ in the intestine is located in cell nuclei (4, 13).

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