EFFECTS OF VITAMIN D ON THE CHANGE OF CYCLIC NUCLEOTIDES AND DEOXYRIBONUCLEIC ACID CONTENTS IN RAT CALVARIA

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Summary The effect of vitamin D on the change of bone contents of cyclic nucleotides and DNA was examined in rats fed a vitamin D-deficient, low calcium diet. Daily administration of vitamin D₃ (0.25 μg) to the animals for a week induced a significant increase of plasma calcium concentration as well as of bone DNA and cyclic AMP contents. After a single administration of vitamin D₃ (0.25 μg), the earliest change observed was the elevation of plasma calcium levels, followed by an increase of the cyclic AMP content which was significant at 48 hr. Significant increase of the DNA content in bone was first observed at 72 hr. In contrast to the increase of cyclic AMP contents in bone, the level of cyclic GMP was almost constant. When the animals were switched to the same vitamin D-deficient, high calcium (3%) diet containing lactose, their serum calcium concentrations were markedly elevated, while neither the cyclic AMP nor DNA contents were changed. These results indicate that although vitamin D administration leads to a change in the cyclic AMP and DNA contents of bone, these changes are not directly related to the early calcium-mobilizing action of the vitamin. The bone change in the cyclic AMP and DNA contents appears to be due to a direct effect of vitamin D rather than to the elevation of serum calcium concentration.

Vitamin D is required to maintain normal bone metabolism and to stimulate bone mineral mobilization in concert with parathyroid hormone (PTH) (1–4). We reported that the level of cyclic AMP in calvaria, both basal as well as PTH-

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stimulated, of vitamin D-deficient, hypocalcemic rats was similar to that of the rats given 2.5 μg of vitamin D₃ 24 hr before, whereas the hypercalcemic effect was observed only in the latter group of the animals (5). It seemed, therefore, that the cause of refractoriness to PTH in vitamin D deficiency was beyond the step(s) after generation of cyclic AMP which has been supposed to be a second messenger of PTH action.

Recently, cyclic nucleotides have been reported to play an important role in regulating cellular proliferation and differentiation (6, 7). Moreover, a marked increase of the level of cyclic GMP was reported in undifferentiated chick oviduct at several hours after steroid hormone administration (8). The vitamin administered to vitamin D-deficient animals also induces the change in bone cell population and increases the number of osteoclasts (9–12).

In the present study, we gave vitamin D to rats fed a vitamin D-deficient, low calcium diet and examined the time course of the change in plasma calcium concentration and bone contents of DNA and cyclic nucleotides. The elevation of plasma calcium levels occurred much earlier than the increase of cyclic AMP and DNA contents in bone.

MATERIALS AND METHODS

Animals. Weanling male rats (Wistar strain) weighing 50g were obtained from a local distributor and maintained on a vitamin D-deficient, low calcium (0.003%) synthetic diet (9, 13) for 2–3 weeks. Some of the rats fed the above low calcium diet for 2 weeks were switched to the same vitamin D-deficient diet which contained high calcium (1.4%), low phosphorus (0.1%) and lactose (20%) (14). Vitamin D₃ (Tokyo Chemical Industry) was administered orally in cotton seed oil.

Assay of cyclic AMP and cyclic GMP in bone. Calvaria dissected out of rats were frozen in dry ice acetone within 30 sec after decapitation and pulverized as previously reported (15). The bone powder of each calvarium (about 50 mg wet tissue) was suspended in 7.5% trichloroacetic acid and a tracer amount of [3H]-cyclic GMP and/or [3H]-cyclic AMP (3,000 cpm of each) was added. The trichloroacetic acid extract neutralized by shaking with ether and adding 1 M Tris was purified on AG 1-X2 (formate forms 0.8–3 cm) column. After washing the column with 1 ml of water, 10 ml of 0.5 N formic acid was first applied to eluate cyclic AMP, followed by 5 ml of 4 N formic acid to eluate cyclic GMP. Each fraction was lyophilized separately and dissolved in 0.5 ml of water. Cyclic AMP was measured by Gilman's binding assay as described previously (16).

Cyclic GMP was assayed using a kit sold by Yamasa Shoyu Co. (Chiba, Japan) which was designed to measure cyclic GMP immunologically after its succinylation. The lower limit of the assay was 100 to 200 femtoles of cyclic GMP per sample, which was sensitive enough to measure cyclic GMP in a calvarium (about 1,000 femtoles). When the cyclic GMP fraction from 50 mg of the tissue purified on AG 1-X2 column was dissolved in 1 ml of 10 mM Tris-Cl solution (pH 7.4) containing 5 mM MgCl₂, the
concentration of cyclic GMP measured was 720 fmole/ml and the twice diluted solution gave a value of 382 fmole/ml. The purified bone extract incubated with 30 mU/ml of phosphodiesterase (Boehringer) at 37°C for 30 min and heated in a boiling water bath for 2 min had no activity of cyclic GMP. When 4,000 fmoles of cyclic GMP was added to 1 ml of the purified extract of 50 mg tissue which contained 720 fmole/ml of cyclic GMP, the measured cyclic GMP was 5,050 fmole/ml and the recovery of added cyclic GMP in the assay was calculated as 107%. These results are presented to verify the validity of the assay.

Measurements of plasma calcium levels and bone contents of DNA. Plasma calcium concentration was determined with an atomic absorption spectrometer (Hitachi-Perkin Elmer 303). The DNA content of calvaria was measured by the method of Burton (17).

RESULTS

Figure 1 shows the effect of vitamin D3, administered in a dose of 0.25 μg/day for a week to rats fed the vitamin D-deficient, low calcium diet, on plasma calcium concentration and bone levels of DNA and cyclic AMP. Vitamin D3 induced significant increases of plasma calcium levels and of both cyclic AMP and DNA contents in bone as expressed on a wet tissue basis. The level of cyclic AMP calculated on DNA content also tended to be higher in the rats repleted with vitamin D3.

![Figure 1](image-url)

Fig. 1. Effects of vitamin D3 on plasma calcium concentration and bone levels of DNA and cyclic AMP. Vitamin D3 was administered orally in a dose of 0.25 μg/day for a week to rats fed the vitamin D-deficient, low calcium diet. The animals were sacrificed 24 hr after the last supplementation of vitamin D3. Each point represents mean ± standard error of at least 4 animals. * Significantly different (<0.05) from vitamin D-deficient animals.
Fig. 2. Time course of the change in plasma calcium concentration (−○−) and bone levels of DNA (−●−) and cyclic AMP (−△−) after vitamin D administration. Animals fed the vitamin D-deficient, low calcium diet for 2 weeks were dosed orally with 0.25 μg of vitamin D₃ and were sacrificed at indicated times thereafter. Each point represents mean ± standard error of at least 4 animals.

Time course in the change of those parameters after a single administration of vitamin D₃ is shown in Fig. 2. Animals fed the vitamin D-deficient, low calcium diet were given a single dose (0.25 μg) of vitamin D₃ and were sacrificed at indicated times thereafter. The earliest change observed was an elevation of plasma calcium levels, followed by an increase of cyclic AMP which was significant at 48 hr after the administration.

Table 1. Time course of the change in cyclic nucleotides in calvaria after a single dose of vitamin D. Animals fed the vitamin D-deficient, low calcium diet for 2 weeks were dosed with 0.25 μg of vitamin D₃ and were sacrificed at indicated times thereafter. Data are expressed as means ± standard errors. Figures in parentheses indicate numbers of animals used.

| Time (hr) | Cyclic AMP (pmole/g wet weight) | Cyclic GMP | cGMP/cAMP |
|-----------|----------------------------------|------------|-----------|
| Vitamin D-deficient (9) After administration of 0.25 μg of vitamin D₃ | 131 ± 10 | 25.0 ± 1.3 | 0.14 ± 0.01 |
| 6 hr (5) | 147 ± 9 | 24.2 ± 1.2 | 0.11 ± 0.01 |
| 12 hr (5) | 138 ± 22 | 24.0 ± 0.8 | 0.13 ± 0.02 |
| 24 hr (9) | 134 ± 25 | 31.4 ± 1.9 | 0.19 ± 0.03 |
| 48 hr (5) | 172 ± 8* | 26.0 ± 1.3 | 0.15 ± 0.01 |
| 72 hr (9) | 255 ± 29* | 23.6 ± 1.7 | 0.12 ± 0.01 |
| 120 hr (5) | 359 ± 52* | 21.7 ± 0.9 | 0.07 ± 0.01* |

* p < 0.05.
administration. A significant increase of DNA content in bone was first observed at 72 hr. In contrast to the increase of cyclic AMP, cyclic GMP content in bone did not show any significant change when calculated on a wet tissue basis (Table 1). When the bone contents of cyclic nucleotides were calculated on a DNA basis, a significant change was observed at 120 hr after vitamin D₃ administration. As compared to those in vitamin D-deficient rats, the cyclic AMP content was increased to $346.9 \pm 54.1$ from $202.3 \pm 15.6$ nmole/mg DNA, whereas the cyclic GMP content was decreased to $20.76 \pm 1.07$ from $40.61 \pm 4.73$ nmole/mg DNA. Such a reciprocal change of cyclic nucleotides lowered the ratio of cyclic GMP/cyclic AMP significantly at 120 hr after vitamin D₃ administration.

Since the elevation of plasma calcium concentration preceded the increases of cyclic AMP and DNA contents in bone after vitamin D administration, the possibility existed that the elevation of plasma calcium induced by feeding the vitamin to vitamin D-deficient rats was responsible for the changes in bone. Administration of a high calcium diet containing lactose to vitamin D-deficient animals previously fed a low calcium diet for 2 weeks, led to a rise in plasma calcium levels to normal, but neither the cyclic AMP nor DNA content of bone showed any change (Table 2). In fact, switching to the high calcium diet lowered the bone content of cyclic AMP significantly, irrespective of the vitamin D status (Table 2).

### DISCUSSION

The skeletal tissue of vitamin D-deficient animals is refractory to the calcium-mobilizing action of PTH, probably as a result of a defect lying beyond the cyclic AMP formation (5). Once vitamin D is given to these animals, the plasma level of calcium is elevated within 24 hr (Fig. 2). As the diet used in the present study

| Calcium (%) | Vitamin D₃ (μg/day) | Plasma Ca (mg%) | DNA (μg/g wet wt.) | Cyclic AMP (pmole/g wet wt.) |
|-------------|---------------------|-----------------|--------------------|-----------------------------|
| 0.003       | 0                   | 4.2 ± 0.1       | 0.64 ± 0.11        | 131 ± 10                    |
|             | 0.25                | 5.9 ± 0.3*      | 1.05 ± 0.06        | 359 ± 52                    |
| 1.4         | 0                   | 10.2 ± 1.2*     | 0.54 ± 0.11        | 73 ± 7                      |
|             | 0.25                | 11.9 ± 0.4*     | 0.86 ± 0.16        | 79 ± 5                      |

* $p<0.05.$
contained an extremely low level of calcium, the source of elevated plasma calcium is probably the skeletal tissue. It also has been shown that the number of bone cells is decreased and osteoclasts are sparse in the bones of vitamin D-deficient hypocalcemic rats, even though the level of circulating PTH is high. An increase in the cell number, especially that of osteoclasts, in rats on the vitamin D repleted, low calcium diet has been observed by Yoshiki et al. (9). This histological observation is in accordance with the finding that the content of DNA in bone is increased at 72 hr after administration of vitamin D.

In considering the action of vitamin D on bone, it seemed most interesting to examine how cyclic nucleotides are involved in the early hypercalcemic effect of vitamin D as well as in the later effect increasing DNA content or bone cell number. When the time course of the change in plasma calcium and cyclic nucleotides is compared (Fig. 2 and Table 2), it is clear that the change in plasma calcium precedes the change in cyclic nucleotides, which excludes a possible role of cyclic nucleotides as a trigger of an early effect of vitamin D initiating bone mineral mobilization. The content of cyclic AMP per wet tissue weight was increased at 48 to 72 hr after vitamin D administration but the change appeared to be in parallel with the increase of DNA content. When the concentrations of cyclic AMP and cyclic GMP were calculated on a DNA basis, a significant increase of cyclic AMP and decrease of cyclic GMP were seen only at 120 hr after vitamin D administration. Thus, it again seems unlikely that the changes of either cyclic AMP or GMP play a role as the trigger to induce an increased level of DNA and probably of bone cell number.

As the elevation of plasma calcium was the earliest detectable effect of vitamin D on bone, it was possible that the mobilization of calcium somehow induced a change in the levels of bone DNA and cyclic nucleotides. However, we could not obtain any evidence to support this notion, as the elevation of plasma calcium levels caused by giving a high calcium diet containing lactose to vitamin D-deficient animals did not increase the levels of either bone cyclic AMP or DNA (Table 2). Rather, feeding the rats with the high calcium diet antagonized the effect of the characteristic action of vitamin D seen in animals fed the low calcium diet. The fact that the effect of vitamin D on the levels of cyclic AMP and DNA was observed only in hypocalcemic rats which must have associated secondary hyperparathyroidism suggests that both vitamin D and PTH are required for these changes in the skeletal tissue. When animals are hypocalcemic, the levels of cyclic AMP versus DNA in both vitamin D-deficient and D repleted rats are higher than those in normo- or hypercalcemic rats (Table 2), probably indicating that the adenylate cyclase activity of bone cells in hypocalcemic rats, even in the absence of vitamin D, is stimulated by the associated hyperparathyroidism. It seems, therefore, that the stimulation of adenylate cyclase in hypocalcemic rats cannot further induce hormone effects unless vitamin D is present, as we suggested previously.

Although we could not obtain evidence to indicate an involvement of cyclic AMP or cyclic GMP in the action of vitamin D to initiate calcium mobilization and DNA synthesis, we feel it is too early to exclude the role of these nucleotides in
vitamin D action. The cyclic nucleotides measured were extracted from tissues which contained heterogenous cells and heterogenous pools of cyclic nucleotides (18–20). It is therefore difficult to detect small changes in these cyclic nucleotide levels, especially in a particular cell type or compartment, which might be critical for the vitamin action. At least such a marked increase of cyclic GMP, as reported in undifferentiated chick oviduct seen at several hours after estrogen administration (8), does not occur in the skeletal tissue, though vitamin D, probably in concert with PTH, undoubtedly triggers a cell proliferation and differentiation in bone.

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