1,25-Dihydroxyvitamin D and not Calcium Is the Major Regulator of Calbindin-D 9-kDa mRNA Levels *In Vivo* (43371)

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Abstract. **A possible role of calcium *in vivo* on intestinal calbindin-D 9-kDa mRNA levels has been studied in rats. In vitamin D-deficient rats, a marked increase in dietary calcium has a small but significant effect on calbindin-D 9-kDa mRNA levels, despite a dramatic increase in serum calcium concentration that clearly resulted from increased intestinal absorption of calcium. On the other hand, vitamin D under all circumstances increased calbindin-D 9-kDa mRNA levels, with the greatest levels found in animals on a low calcium diet where little or no calcium is available for absorption. These results strongly support the idea that 1,25-dihydroxyvitamin D is directly responsible for the induction of calbindin-D 9-kDa.** [P.S.E.B.M. 1992, Vol 199]

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) plays a central role in calcium homeostasis in part by stimulating calcium absorption. The mechanism by which 1,25-$(OH)_2D_3$ stimulates intestinal calcium and phosphorus absorption is not yet understood. In mammals, a 9-kDa calcium-binding protein is induced in the intestine in response to 1,25-$(OH)_2D_3$ (1). Both the cDNA and gene for the rat 9-kDa protein have been sequenced (2–5). Thus far, no firm evidence of a vitamin D-responsive element in the gene has been obtained, despite intense efforts. Of great interest is the demonstration that calcium in culture can induce calbindin-D 9-kDa or calbindin-D 28-kDa (6, 7). This finding could provide an explanation of how 1,25-$(OH)_2D_3$ can induce the calbindin-Ds, namely, that calcium entering the enterocyte as a result of increased calcium absorption could induce the vitamin D-dependent calbindins. We have examined this possibility *in vivo* in young growing rats. Our results do not support the idea that calcium induces calbindin-D 9-kDa *in vivo*, but supports the idea that 1,25-$(OH)_2D_3$ itself is the inducer (6, 7).

Methods

Animals. Male Sprague-Dawley rats (Harlan Sprague-Dawley Co., Madison, WI) were obtained as weanlings and maintained on a vitamin D-deficient diet containing 0.47% calcium and 0.3% phosphorus supplemented with vitamins A, E, and K (8). After 5 weeks, they were shifted to the modified diets as indicated for 12 days. Serum calcium levels were analyzed by atomic absorption spectrophotometry.

Buffers. The following buffers were used: MOPS: 0.2 *M* morpholinepropanesulfonic acid (pH 7.0), 50 m*M* sodium acetate, 5 m*M* EDTA; SSC, 20× standard saline citrate: 3.0 *M* NaCl, 0.9 *M* sodium citrate (pH 7.0); and 50× Denhardt's solution: 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin (Fraction V).

RNA Isolation. Total RNA was isolated from duodena by homogenization in 4 *M* guanidine thiocyanate, 5 m*M* sodium citrate (pH 7.0), 0.1 *M* 2-mercaptoethanol, and 0.5% sarcosyl using a Polytron (Brinkmann Instruments, Westbury, NY), followed by cesium chloride density gradient centrifugation (9). The RNA was quantitated by measurement of absorbance at 260 nm, and adjusted to 2 mg/ml in diethyl pyrocarbonate (Sigma, St. Louis, MO) -treated water.

Northern Gel Analysis. Total RNA (10 μg) was

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electrophoresed on a 1% agarose gel in 1.1% formaldehyde and 1× MOPS, and transferred to nitrocellulose membrane (Biotrace NT; Gelman Sciences, Ann Arbor, MI) according to standard procedures (9). The membrane was prehybridized in 6× SSC, 1× Denhardt's solution, 0.1% sodium dodecyl sulfate, and 100 μg/ml of sheared and denature salmon sperm DNA at 42°C for 4 hr. Two radiolabeled oligonucleotides were used as probes, as described previously (4). The gel was visualized by autoradiography using X-OMAT AR film (Kodak) exposed for 1 day with a Cronex Lightning Plus intensifying screen (DuPont/NEN). The radiolabel was quantitated by a Beta particle detection device (Betagen, Waltham, MA) to confirm the slot blot analysis. A lactic dehydrogenase cDNA probe was used as a reference for RNA loading and, in all cases, values reported were corrected as described previously (10).

Slot Blot Analysis. Total RNA (20 μg) was measured by a slot blot hybridization assay (Minifold II; Schleicher and Schuell, Inc., Keene, NH). The membrane (Hybond-N; Amersham) was prehybridized and hybridized as in the Northern blot analysis. Autoradiographs were scanned with a soft laser-scanning densitometer model SL-504-XL (Biomed Instruments, Fullerton, CA). There were at least five animals in each group, which resulted in at least five determinations for each value presented. Statistical analysis was by Student's *t* test.

Results

Figure 1 compares the levels of serum calcium and calbindin-D 9-kDa mRNA levels of animals kept on the various diets. Animals on a vitamin D-deficient diet with normal levels of calcium (0.47%) and phosphorus (0.3%) show a low level of calbindin-D 9-kDa mRNA and are hypocalcemic. The addition of 25 units/day of vitamin D to this diet results in a 4.5-fold increase of calbindin-D 9-kDa mRNA levels and a correction of the hypocalcemia. Animals switched to a high calcium diet (1.2%) showed normal serum calcium levels and a slight but significant increase in calbindin-D 9-kDa mRNA levels. The increased serum calcium could only have resulted from an increased intestinal absorption. A decrease in dietary phosphorus from 0.3% to 0.1% caused a slight and insignificant elevation in serum calcium levels and did not significantly increase calbindin-D 9-kDa mRNA above that seen with the 1.2% calcium-0.3% phosphorus diet. Animals fed a low calcium diet (0.02%) supplemented with vitamin D showed the highest level of calbindin-D 9-kDa mRNA. Since there is virtually no calcium in the intestine in this case, vitamin D could not have increased calcium flux across the enterocytes in this case to the level occurring with a 0.47% calcium diet. These animals had subnormal serum calcium levels (8.8 mg/100 ml), while the calbindin-D 9-kDa mRNA level was increased 7.2-fold over animals on a normal calcium and phosphorus vitamin D-deficient diet. In every case, regardless of the calcium and phosphorus levels, vitamin D always increased calbindin-D 9-kDa mRNA to between 4.5- and 7.2-fold over control.

Figure 2 shows a Northern blot depicting the dependence of calbindin-D 9-kDa mRNA induction on vitamin D. Lane 1 shows the level of calbindin-D 9-kDa mRNA levels in animals fed a vitamin D-deficient diet with normal calcium and low phosphorus. Lane 2 is RNA from animals fed a vitamin D-deficient diet with low calcium and normal phosphorus. Lane 3 is RNA from animals fed a vitamin D-deficient diet with high calcium and normal phosphorus. Lanes 4 and 5 are RNA from animals fed a vitamin D-sufficient diet with normal phosphorus and either low calcium (Lane 4) or high calcium (Lane 5), respectively. Finally, Lane

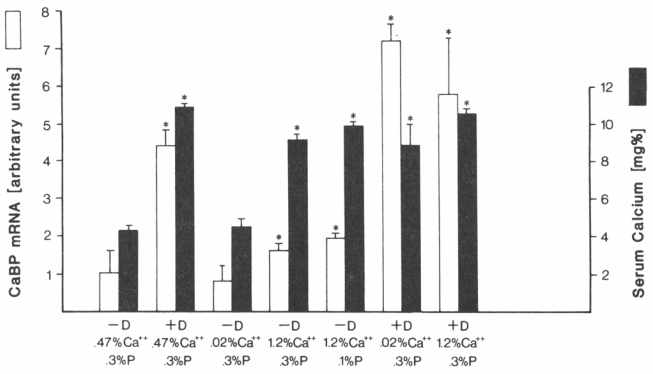

Figure 1. Dietary effects of calcium, phosphorus, and vitamin D on serum calcium and calbindin-D 9-kDa mRNA levels as revealed by density measurements of autoradiographs of Northern gel analyses. Animals were kept on a vitamin D-deficient diet for 5 weeks and then switched to the indicated diets for 12 days ($n = 3$). Asterisks indicates a statistically significant difference between test groups as compared with a vitamin D-deficient diet containing 0.47% calcium and 0.3% phosphorus group as the control ($P < 0.05$).

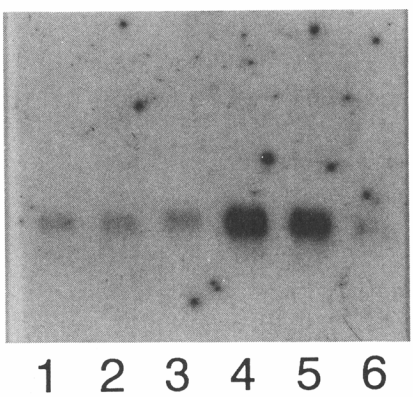

Figure 2. Northern analysis of 10 μg of total RNA isolated from rats on the following diets: Lane 1, vitamin D deficient, 0.47% calcium, 0.1% phosphorus; Lane 2, vitamin D deficient, 0.02% calcium, 0.3% phosphorus; Lane 3, vitamin D deficient, 1.2% calcium, 0.3% phosphorus; Lane 4, vitamin D supplemented, 0.02% calcium, 0.3% phosphorus; Lane 5, vitamin D supplemented, 1.2% calcium, 0.3% phosphorus; and Lane 6, vitamin D deficient, 0.47% calcium, 0.3% phosphorus. The above experiment was repeated on three different occasions.

6 is RNA from animals fed a vitamin D-deficient diet with normal calcium (0.47%) and phosphorus (0.3%) levels. In these cases, the RNA loaded per lane was equivalent, as indicated by a lactic dehydrogenase cDNA probe on the same gel as described earlier (10). Quantitation of the Northern gel using a Betagen scanner correlated exactly with the results seen in Figure 2.

Discussion

It is possible even in the absence of vitamin D to markedly increase calcium absorption in the rat by simply raising dietary calcium levels, although this may be by a paracellular route (11, 12). Thus, it is possible to ask the question of whether calcium can induce calbindin-D 9-kDa in the absence of vitamin D. In agreement with all previous work, our results show that by increasing dietary calcium from 0.47% to 1.2% to a vitamin D-deficient rat increases serum calcium from 4.2 mg/100 ml to 10.5 mg/100 ml. This increase is clearly from increased absorption and, hence, flux across or around the enterocytes. This flux of calcium failed to cause an increase in calbindin-D 9-kDa mRNA levels. On the other hand, vitamin D given to rats on a diet virtually devoid of calcium gave a 7.2-fold increase in calbindin-D 9-kDa mRNA. Since there was virtually no calcium in the diet, vitamin D in this case could not have increased the flux of calcium across the enterocyte (11–13). The rise in serum calcium in this case is clearly at the expense of bone (13, 14). These results show that *in vivo* vitamin D presumably through $1,25\text{-}(OH)_2D_3$ is responsible for inducing transcription of the calbindin-D 9-kDa gene.

Certainly, Brehier *et al.* (6) could induce calbindin-D 9-kDa transcription *in vitro* with calcium in embryonic rat intestine. Clemens *et al.* (7) similarly demonstrated that calcium in culture can also increase the 28-kDa calbindin-D mRNA. However, *in vivo*, apparently induction by calcium does not take place to a significant degree, as evidenced by these experiments or those of Wasserman and Taylor (15) and Buckley and Bronner (16). These results direct attention to the mechanisms whereby $1,25\text{-}(OH)_2D_3$ and its receptor induce transcription of the calbindin-D 9-kDa and 28-kDa genes, rather than to a mechanism in which calcium serves in some way as the inducer. However, the small but significant increase in calbindin-D 9-kDa mRNA caused by the high dietary calcium and serum calcium in the absence of vitamin D suggests a possible minor role of calcium in modulating calbindin-D 9-kDa transcription. Calcium could also play a role at the translational level which was not addressed here. Our results most definitely point to a major role of $1,25\text{-}(OH)_2D_3$ itself in the transcriptional regulation of the calbindin-D 9-kDa gene.

It is noteworthy that even in animals on a vitamin D-deficient diet, substantial amounts of calbindin-D 9-kDa mRNA are detectable in total RNA preparations (Fig. 2). Although these animals were severely hypocalcemic and had undetectable levels of both $1,25\text{-}(OH)_2D_3$ and $25\text{-OH-}D_3$, the calbindin-D 9-kDa gene is still significantly expressed. This might be due to a constitutive promoter of the calbindin-D 9-kDa gene or there may exist as yet unidentified factors that regulate its expression.

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