Stimulation in vitro by 1,25-Dihydroxy-Vitamin D_3 of Intestinal Cell Calcium Uptake and Calcium-Binding Protein

Abstract. *Treatment of duodenal tissue from rats deficient in vitamin D with 1,25-dihydroxy-vitamin D_3 [1,25-$(OH)_2$-D_3] led to more than a doubling of calcium uptake by the isolated cells and the appearance in those cells of previously undetectable calcium-binding protein (CaBP). Treatment with the precursor, 25-hydroxy-vitamin D_3, was without effect on calcium uptake or CaBP. Cells from vitamin D–replete animals took up three and a half times more calcium than cells from deficient animals. This rapid (90-minute) effect of in vitro treatment with a physiological dose (4.7×10^{-8}M) of 1,25-$(OH)_2$-D is the first such report and is in accord with the regulatory role of the hormone-like sterol.*

Several investigators have used isolated intestinal cells (*1*) or villi (*2*) to study calcium transport, but did not find that calcium uptake by their preparations mimicked calcium absorption. This led Bray and Clark (*3*) to conclude that such preparations are unphysiological and that calcium uptake represents binding of questionable significance. We now report that isolated intestinal cells can be made to take up calcium under conditions that suggest that the cell uptake process reflects events in vivo and is indeed a metabolic event.

Table 1. Calcium uptake of isolated duodenal cells from vitamin D–deficient and vitamin D–replete rats. Calcium uptake (picomoles per 10^6 cells) refers to the values at the end of a 5-minute incubation period, with the time course of all experiments like that in Fig. 1.

Experiment (No.)	Animal cells	Addition in vitro*	Ca uptake (mean ± S.E.)	Ratio
3	D–replete		190.2 ± 12.5†	3.5
	D–deficient		54.2 ± 15.4	
7	D–deficient	$4.7 \times 10^{-8}M$ 1,25-$(OH)_2$-D_3	89.3 ± 13.9†	2.6
	D–deficient	Vehicle	35.0 ± 5.1	

*In vitro addition refers to inclusion in the isolation medium (see legend, Fig. 1). †Significantly greater ($P < .05$) than their respective controls, with "ratio" the ratio of uptake of the two groups.

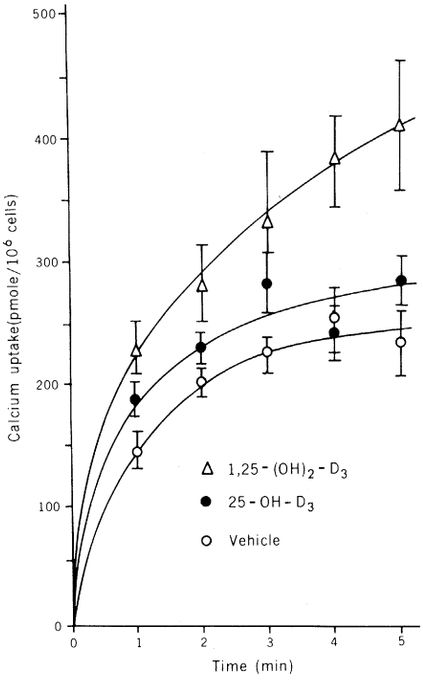

Fig. 1. Effect in vitro of 1,25-dihydroxy-vitamin D_3 or 25-hydroxy-vitamin D_3 on calcium uptake by rat duodenal cells. Vitamin D–deficient, hypocalcemic, male Sprague-Dawley rats (140 g) were decapitated; the duodenum was excised, slit lengthwise, rinsed in cold saline, and cut into 1- to 2-cm pieces. The pieces were randomly distributed into 50-ml plastic erlenmeyer flasks containing 20 ml of Tyrode's salt solution (Ca- and Mg-free) with 0.1 percent bovine serum albumin (BSA) and 0.1 percent hyaluronidase (HSE, Worthington Biochemical Corp.). The flasks contained either 15 units (0.39 μg, $4.7 \times 10^{-8}M$) 1,25-dihydroxy-vitamin D_3, or 15 units of 25-hydroxy-vitamin D_3, or an equal volume of ethanol and propylene glycol (1 : 1) (vehicle). Incubation was for 90 minutes at 37°C with shaking and a continuous supply of oxygen. Intestinal debris was removed by filtration through gauze. The filtrate was centrifuged at 4°C (≃400*g*, 10 minutes), and the sedimented cells were washed twice with ice-cold Ca- and Mg-free Tyrode's salt solution, containing 0.1 percent BSA, and resuspended to a concentration of ≃ 10^7 cell/ml. Cell number and viability were determined by adding trypan blue (0.02 percent final concentration) to a portion and counting the number of viable cells (those that excluded the dye). Preparations with viable cells in excess of 70 percent were assayed as follows: 25 μl of the cell suspension were distributed into tubes kept at 21°C; 15 μl of tris-saline buffer (0.0137*M* tris-HCl, 0.12*M* NaCl, 3 m*M* KCl, *p*H 7.4) and 10 μl of ^{45}Ca (≃ 0.4 μc corresponding to ≃ 0.9×10^6 c/mole) were added; the samples were shaken and oxygenated, and the reaction was stopped 1, 2, 3, 4, and 5 minutes later by the rapid addition of 2 ml of tris-saline buffer, followed at once by filtration through Millipore disks (HA, 0.45 μm, 25 mm in diameter). Each disk was washed once more with 2 ml of tris-saline buffer and then counted by liquid scintillimetry [Bray's solution (*21*)]. The calcium concentration in the assay was 40 μ*M*. Typical counts for the 5-minute points were 27×10^3 and 33×10^3 for the cells treated with 1,25-$(OH)_2$-D_3, and 17×10^3 and 20×10^3 for the cells treated with vehicle.

Cells were harvested from male Sprague-Dawley rats that had been weaned and maintained on partially synthetic diets (*4*). The diets contained either no vitamin D_2 or 2200 international units of vitamin D_2 per kilogram of feed. Calcium and phosphorus content was determined analytically (*5*); vitamin D content was verified by Warf Institute, Inc., Madison, Wisconsin. Animals were considered deficient in vitamin D when they became hypocalcemic [< 7 mg of Ca per 100 ml of plasma (*5*)], or when their intestines, as shown in parallel experiments (*6*), contained undetectable amounts of the vitamin D–dependent calcium-binding proteins (CaBP) (*6, 7*), or both.

Cells were isolated and assayed (Fig. 1). The isolation is a modification of the procedures described by Perris (*8*) and Kimmich (*9*), and the assay is an adaptation of a Millipore filtration procedure (*10*). Microscopic examination of the isolated cells revealed the typical appearance of intact mucosal cells. However, the number of intact cells isolated varies and affects the absolute calcium uptake. For this reason the cells assay concentration was standardized at 10^7 cell/ml.

The calcium uptake of cells isolated from vitamin D–deficient hypocalcemic cells increased with time and addition to the isolation medium of $4.7 \times 10^{-8}M$ 1,25-dihydroxy-vitamin D_3 [1,25-$(OH)_2$-D_3] (*11*) markedly and significantly stimulated calcium uptake. This was not the case when a similar amount of 25-hydroxy-vitamin D_3 (25-OH-D_3) (*12*) was added to the isolation medium. The time course and assay errors (~ 10 percent of the standard error) shown in Fig. 1 are typical of the experiments reported. 25-OH-D_3 is the metabolite of vitamin D_3 that is made in the liver (*13*) and is transformed in the kidney (*14, 15*) to the active metabolite, 1,25-$(OH)_2$-D_3.

In seven experiments the addition of this active metabolite to the isolation medium on the average led to a 2.6-fold increase in calcium uptake in duodenal cells isolated from vitamin D–deficient animals (Table 1). Moreover, when the calcium uptake was compared between cells from vitamin D–deficient and from vitamin D–replete animals, cells from the latter took up 3.5 times more calcium on the average than cells from the deficient animals. Thus treatment in vitro with 1,25-$(OH)_2$-D_3 raised cellular calcium uptake to about three-fourths that found in naturally replete cells. In a further test of the vitamin D–related increase in calcium uptake we administered to vitamin D–deficient hypocalcemic animals 15 units (0.39 μg) of 25-OH-D_3 by intraperitoneal injection. The

duodenal cells were then harvested and assayed approximately 30 hours later. At that time the cells from the treated animals showed a 3.1-fold increase in calcium uptake, as compared with the cells from the deficient controls. This experiment also demonstrates that our preparation of 25-OH-D_3 was biologically active. The 30-hour interval was chosen on the basis of prior work (*6, 16*).

We wondered whether the increased calcium uptake due to 1,25-$(OH)_2$-D_3 was associated with the appearance of CaBP. Cells from vitamin D–replete and vitamin D–deficient animals were homogenized at the end of the uptake experiment, and CaBP was fractionated and assayed (Fig. 2). The isolation procedure and assay have been shown (*7*) to detect the vitamin D–dependent CaBP and to measure its specific activity quantitatively. The Chelex assay agrees with immunological measurements for chick CaBP (*17*). CaBP was found [$V_e/V_o = 2$, where V_e is the elution volume, 68 ml (Fig. 2) and V_o is the void volume, 34 ml (Fig. 2)] (*7*) in the material from the vitamin D–replete animals (Fig. 2, + D), was absent in the material from the deficient animals (Fig. 2, –D) (*6, 7*), and was found in the 1,25-$(OH)_2$-D_3–treated material from the deficient animals (–D plus 1,25-$(OH)_2$-D_3) (Fig. 2). The amount of CaBP cells treated in vitro was two-thirds of that found in replete cells, comparable to the increase in calcium uptake. In a separate experiment we were unable to detect CaBP in material from deficient animals whose duodenal cells had been isolated in the presence of 25-OH-D_3, but were able to detect CaBP in cells derived from deficient animals that had been treated with 25-OH-D_3 30 hours before assay.

Corradino (*18*) has reported the induction of intestinal CaBP in a chick organ culture system in 6 to 12 hours, but this is the first report of CaBP having been found after treatment in vitro that lasted less than 3 hours. The finding of CaBP in 1,25-$(OH)_2$-D_3–treated material from vitamin D–deficient rats points to a metabolic action of 1,25-$(OH)_2$-D_3 in our system. At the same time it lends further support to a possible role of CaBP in transepithelial calcium movement. The relatively short time required to demonstrate previously undetectable CaBP may be related to the higher metabolic turnover of mammalian as compared to avian cells. Precise mechanisms are as yet unknown.

Inhibitors of oxidative phosphorylation depressed calcium uptake by about half, but the two compounds that bind -SH groups had no effect (Table 2). This suggests that at least part of the calcium uptake measured here requires metabolic activity. It is also consistent with a direct mitochondrial involvement (*19*).

Table 2. Effect of metabolic inhibitors on calcium uptake by isolated rat duodenal cells. Cells were obtained from vitamin D–deficient hypocalcemic rats (see legend, Fig. 1). After isolation cells were assayed in the absence or presence (5 m*M* final concentration) of inhibitor and the reaction stopped after 3 minutes.

Inhibitor	Ca uptake (pmole/10^6 cell)		Inhibition* (%)
	Exp. 1	Exp. 2	
Control	285	323	
Dinitrophenol	128	125	58 ± 3
Methylene blue	128	157	53 ± 2
Potassium cyanide	152	144	51 ± 4
Dichlorophenol indophenol	136	173	49 ± 3
p-Chloromercuribenzoate	269	304	6 ± 1
Iodoacetamide	275	315	3 ± 1

*Mean ± the standard error.

Thus three lines of evidence support the inference that at least some of the cellular calcium uptake as measured here represents a metabolic event that may be related to active calcium transport in vivo. (i) Cells from vitamin D–deficient animals took up less calcium than cells from replete animals. (ii) Treatment in vitro of intestinal tissue from vitamin D–deficient animals with 1,25-$(OH)_2$-D_3, but not with its precursor 25-OH-D_3, caused the isolated duodenal cells to take up 2.6 times as much calcium as control cells and led to the appearance of CaBP, a protein thought to be associated with active calcium transport. (iii) Treatment of cells in vitro with inhibitors of oxidative phosphorylation led to a halving of calcium uptake. Of these, the most compelling and novel is the response of cells from vitamin D–deficient animals to in vitro treatment with 1,25-$(OH)_2$-D_3.

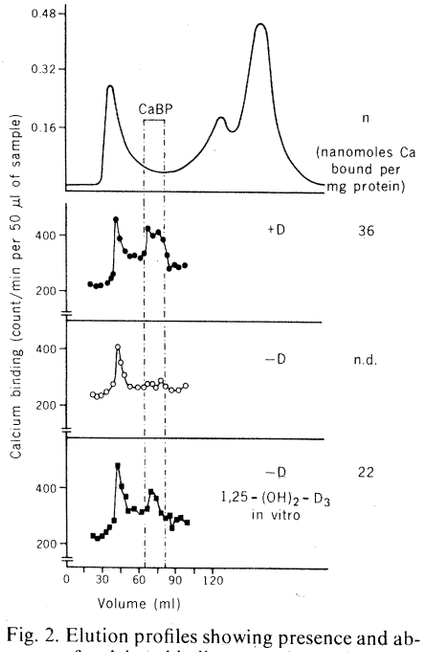

Fig. 2. Elution profiles showing presence and absence of calcium-binding protein. Isolated intestinal cells were homogenized and centrifuged (100,000*g*, 60 minutes); 10 mg of the supernatant protein was lyophilized. The sample was taken up in 0.5 ml elution buffer (0.02*M* ammonium acetate, 1 m*M* mercapoethanol, *p*H 7.2) and chromatographed on a Sephadex G-50 column (1.5 by 30 cm). Protein was monitored at 280 nm (top panel) and calcium binding determined quantitatively by a competitive Chelex resin assay (*7*). Abbreviations: n, nanomoles of Ca bound per milligram of protein refers to the maximum amount of calcium binding associated with the vitamin-D dependent calcium-binding protein peak (CaBP) (*7*); n.d., not detectable.

The rapid appearance of CaBP in these experiments is consistent with a regulatory role (*15*) of the hormone-like sterol. In a more general sense our approach promises to be of use also in the study of other transport deficiency diseases (*21*).

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11. We thank Dr. A. W. Norman, Riverside, California for the gift of the 1,25-dihydroxy-vitamin D_3 used in these experiments.
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Lipoprotein and Lecithin : Cholesterol Acyltransferase Changes in Galactosamine-Induced Rat Liver Injury

Abstract. *Abnormal lipoproteins and decreased lecithin : cholesterol acyltransferase activity are found in rat plasma following intraperitoneal injection of* D-*galactosamine. The changes observed, including absence of* α*-lipoprotein and the presence of lipoproteins rich in phospholipid and unesterified cholesterol but deficient in cholesteryl esters, are remarkably similar to changes found in human pathologic states of lecithin : cholesterol acyltransferase deficiency. When examined by electron microscopy, all of the major lipoprotein classes isolated by ultracentrifugation showed morphological abnormalities including the formation of rouleaus consisting of disk-shaped particles.*

Lecithin : cholesterol acyltransferase (LCAT), an enzyme found in the plasma of many animal species, catalyzes the transesterification of an unsaturated fatty acid from the 2-position of phosphatidyl choline (lecithin) to the 3-position of cholesterol. In man, nearly all of the esterified cholesterol in plasma is derived from this reaction (*1*). It has been postulated that this enzyme plays a vital role in the metabolism of plasma lipoproteins by facilitating the exchange of unesterified cholesterol and cholesteryl esters between plasma lipoproteins, since profound abnormalities in the composition, ultrastructure, and metabolism of lipoproteins have been demonstrated in familial LCAT deficiency (*2*). Abnormal lipoproteins have also been reported in other diseases in which plasma LCAT deficiency occurs, including liver disease (*3*), abetalipoproteinemia (*4*), and Tangier's disease (α-lipoprotein deficiency) (*5*). Despite these observations, the exact nature of the role of LCAT in cholesterol and lipoprotein metabolism is not clear nor has the relationship of deficiency in LCAT activity to specific abnormalities in lipoprotein composition and structure been determined.

In rats, intraperitoneal injection of D-galactosamine produces liver injury secondary to uridylate trapping in the form of uridine diphosphate hexosamines (*6*). Single injections of D-galactosamine (350 to 750 mg per kilogram of body weight), which result in minimal injury, cause accumulation of hepatic triglycerides without a decrease in plasma lipids as found with other inhibitors of protein synthesis that produce a fatty liver (*7*). The reported decrease in rat plasma LCAT activity following D-galactosamine injection (*8*) and the results of our own experiments (*7*) suggest that the D-galactosamine model can provide a unique means of investigating the regulation of cholesterol esterification by LCAT and the role of this enzyme in lipoprotein metabolism. In the studies reported here we demonstrate that D-galactosamine produces striking compositional and morphological changes in all classes of plasma lipoproteins associated with a rapid and reversible LCAT deficiency.

Female Sprague-Dawley rats, 180 to 200 g, received a single intraperitoneal injection of D-galactosamine hydrochloride (750 mg/kg) dissolved in isotonic saline. Blood samples were taken from the abdominal aorta of ether-anesthetized animals. Rats that were bled 24 hours after injection were fasted from the time of injection. Animals bled at other times were fasted at least 12 hours but no more than 24 hours before blood was drawn. Controls that were fasted for times equal to the treated animals were used for comparison. LCAT activity was defined as the incorporation of [^{14}C]cholesterol, equilibrated with plasma cholesterol, into cholesteryl ester (*9*). In some experiments LCAT activity was also measured as initial rate of disappearance of unesterified cholesterol as determined by gas chromatography. The results were essentially the same. Total cholesterol and unesterified cholesterol were determined by gas chromatography of extracts of saponified and nonsaponified samples (*10*).

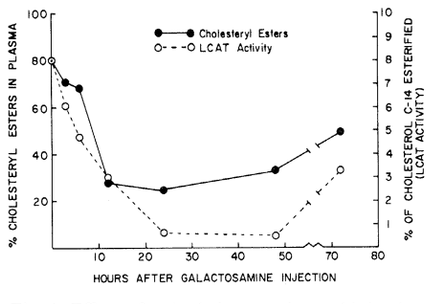

Fig. 1. Effect of a single intraperitoneal injection of D-galactosamine hydrochloride (750 mg/kg) on cholesteryl esters and lecithin : cholesterol acyltransferase (LCAT) in rat plasma. Cholesteryl esters are represented as percentage of total plasma cholesterol.

The effect of a single dose of D-galactosamine on plasma LCAT activity and on the proportion of total cholesterol present in the esterified form is shown in Fig. 1. Within 3 hours there is a 25 percent decrease in LCAT activity, and this decrease continues until 24 hours, when LCAT activity is less than 10 percent of control levels. During this same period the total plasma cholesterol is essentially constant (61 mg/dl), which means that the decrease in cholesteryl esters is accompanied by an increase in unesterified cholesterol. The LCAT activity remains at this level at 48 hours but has begun to increase again by 72 hours. Parallel to the decreased LCAT activity there is a concomitant decrease in the percentage of esterified cholesterol in plasma, which reaches a minimal value by 12 hours and begins to recover after 48 hours. This effect of D-galactosamine on LCAT activity is not a direct effect on the LCAT reaction, since the direct addition to normal rat plasma of more than five times the theoretically possible plasma concentration of D-galactosamine that could result from the dose has no effect on LCAT activity.

The plasma lipoproteins from these animals were separated by agarose electrophoresis and stained with Fat Red 7B (*11*). Within 3 hours the α-lipoprotein is decreased and is completely absent at 24 and 48 hours (Fig. 2). After 3 hours the pre-β-lipoprotein [very low density lipoprotein (VLDL)] has disappeared and the β-lipoprotein has a greater mobility. These changes progress until the only remaining lipoprotein at 24 hours is a band that stretches from the point of application (origin) to approximately the pre-β position and by 48 hours this single band is even further diminished. By 72 hours all of the lipoproteins have reappeared; however, the β-lipoprotein band, which is normally low in fasted rats, is increased, and the α-lipoprotein band is still decreased compared to the control.

Lipoproteins from a pool of plasma obtained from rats 24 hours after injection of