

1,25 Dihydroxyvitamin D Increases Hepatocyte Cytosolic Calcium Levels

A Potential Regulator of Vitamin D-25-Hydroxylase

Daniel T. Baran and Moira L. Milne

Departments of Orthopedics and Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01605

Abstract

1,25 dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$) has been demonstrated to inhibit hepatic 25 hydroxyvitamin D (25 OHD) production. Changes in cytosolic calcium have been shown to regulate cellular processes. Using the fluorescent dye Quin 2, we have investigated the effects of $1,25(\text{OH})_2\text{D}$ and $24,25(\text{OH})_2\text{D}$ on cytosolic calcium levels in hepatocytes. $1,25(\text{OH})_2\text{D}$ exposure for 5 min increases cytosolic calcium levels by 24% at a concentration of 100 pg/ml, 39% at a concentration of 1 ng/ml, and 50% at a concentration of 2 ng/ml. The latter increment occurs in both the presence and absence of extracellular calcium, indicating that $1,25(\text{OH})_2\text{D}$ is mobilizing intracellular calcium pools. $24,25(\text{OH})_2\text{D}$, 10 ng/ml, does not increase cytosolic calcium levels while the calcium ionophore A23187, 3 μM , increases levels by 52%. Calcium inhibits hepatic 25 OHD synthesis in liver homogenates in a dose-dependent fashion, which can be prevented by chelation of calcium with EGTA. $1,25(\text{OH})_2\text{D}$ and A23187 decrease hepatocyte 25 OHD synthesis. The inhibitory effect of A23187 can be prevented by chelation of extracellular calcium. The data demonstrate that $1,25(\text{OH})_2\text{D}$ increases hepatocyte cytosolic calcium, and that these increments in cytosolic calcium may regulate some of the hepatic actions of the vitamin D metabolite.

Introduction

Current evidence suggests that the calcium ion is an intracellular messenger in hormone action and that alterations in cytosolic calcium concentrations are instrumental in hormone synthesis and secretion. Calcium is known to play an important role in insulin release (1, 2) and recent reports indicate that 1,25 dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$)¹ is essential for normal insulin secretion in the perfused rat pancreas (3), suggesting a possible relationship between $1,25(\text{OH})_2\text{D}$ and intracellular calcium levels.

Recent studies have begun to examine the regulation of 25 hydroxyvitamin D (25 OHD) and the effect of disease on this process. $1,25(\text{OH})_2\text{D}$ inhibits 25 OHD production by the per-

fused rat liver and rat liver homogenates within 2 h (4). Inhibition of 25 OHD synthesis by $1,25(\text{OH})_2\text{D}$ is greater in the perfused liver (45%) than in liver homogenates (11%), suggesting that the $1,25(\text{OH})_2\text{D}$ effect is augmented in the intact system (4). Circulating $1,25(\text{OH})_2\text{D}$ levels also inhibit hepatic 25 OHD production in humans. Administration of $1,25(\text{OH})_2\text{D}$ to postmenopausal women with osteoporosis is attended by a significant decrease in circulating levels of 25 OHD (5). Likewise, treatment of normal subjects with $1,25(\text{OH})_2\text{D}$ prevents an increase in serum 25 OHD levels in response to a challenge with vitamin D (6).

We have investigated the effects of $1,25(\text{OH})_2\text{D}$ on hepatocyte cytosolic calcium levels, and of calcium on hepatic 25 OHD production. The results indicate that (1) the vitamin D metabolite increases hepatocyte cytosolic calcium levels in a dose-dependent fashion; (2) the calcium ion inhibits 25 OHD synthesis by rat liver homogenates also in a dose-dependent fashion; and (3) the calcium ionophore which also increases cytosolic calcium inhibits hepatocyte 25 OHD production. The data suggest that the $1,25(\text{OH})_2\text{D}$ -induced increments in cytosolic calcium may mediate some of the actions of this vitamin D metabolite.

Methods

Animal maintenance. All rats are housed in the vivarium at the University of Massachusetts Medical Center. When vitamin D-deficient animals are required, 3-wk-old male weanling rats are housed in the absence of ultraviolet light and fed a vitamin D-deficient diet (Teklad, Madison, WI) containing 0.2% calcium and 0.4% phosphate for 8 wk. At that time blood levels of 25 OHD are 0.8 ± 0.3 ng/ml, $1,25(\text{OH})_2\text{D}$ levels 20 ± 3 pg/ml, calcium 5.9 ± 0.2 mg/dl, and phosphorus 10.5 ± 0.6 mg/dl.

Hepatocyte preparation. The liver is perfused for 30 min in the presence of collagenase (Cooper Biomedical, Inc., Malvern, PA), 0.15 mg/ml, in a perfusate buffer system containing 2.5% washed red blood cells and 97.5% Hepes-buffered salt solution (BSS) without calcium or magnesium. The temperature in the perfusion chamber is 37°C with a humidity of 90–93%. The pO_2 of the perfusion solution is maintained at 100 mm Hg by a constant flow of 95% O_2 /5% CO_2 (5 liters/min) through the "lung" assembly within the perfusion chamber. Details of the surgical procedure are given elsewhere (7).

After taking the liver from the perfusion chamber, the connective tissue is removed and the liver sliced into small pieces in serum-free Hepes-BSS containing 1.5 g/dl albumin. The BSS contains NaCl 124 mM, NaHCO_3 18.3 mM, KCl 5 mM, KH_2PO_4 1 mM, $\text{MgSO}_4/7\text{H}_2\text{O}$ 1 mM, $\text{CaCl}_2/\text{H}_2\text{O}$ 1.8 mM, and glucose 11 mM with a pH of 7.4 at 37°C . The calcium ion concentration is 0.5 mM. The homogenate is filtered through fine mesh and centrifuged at 50 g for 1 min. The pellet is resuspended in the Hepes-BSS at 37°C , washed, and recentrifuged a total of three times. The cell viability is determined by trypan blue dye exclusion. The viability for vitamin D-deficient hepatocytes is 60–72%, comparable to the 58% viability reported by others (8), yielding $20\text{--}40 \times 10^7$ viable hepatocytes/liver.

Determination of cytosolic calcium levels by Quin 2 fluorescence. Hepatocytes, 5×10^7 cells, are incubated in a water bath for 10 min in 5 ml of Hepes-BSS containing 1.5 g/dl albumin at 37°C under 95% O_2 /

Address correspondence to Dr. Baran.

Received for publication 3 June 1985 and in revised form 3 February 1986.

1. *Abbreviations used in this paper:* 25 OHD, 25 hydroxyvitamin D; $1,25(\text{OH})_2\text{D}$, 1,25 dihydroxyvitamin D; $24,25(\text{OH})_2\text{D}$, 24,25 dihydroxyvitamin D; BGJ, Biggers-Gwatkin-Judah; buffered salt solution; DMSO, dimethylsulfoxide; F_{max} , maximal fluorescence; F_{min} , minimal fluorescence; HPLC, high performance liquid chromatography.

J. Clin. Invest.

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0021-9738/86/05/1622/05 \$1.00

Volume 77, May 1986, 1622–1626

5% CO₂ at 60 oscillations/min. Quin 2AM (CalBiochem-Behring Corp., San Diego, CA), 500 nmoles, in 5 μ l of DMSO, is added to 5 ml of cells for 15 min. The cells are diluted to 15 ml with the Hepes-BSS (without albumin) and centrifuged at 50 g. They are resuspended in 5 ml of Hepes-BSS (without albumin) and incubated for an additional 15 min.

Cellular fluorescence is determined in a 650-10S Scanning Fluorescence Spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). Fluorescence readings (*F*) are taken at an emission wavelength of 495 nm and a slit width of 5 nm, and an excitation wavelength of 339 nm and a slit width of 5 nm with constant stirring (Spectrocell, Inc., Orelan, PA). The emission spectrum is determined by scanning between an emission of 380 nm and 530 nm. Recordings are made on an R100A recorder (Perkin-Elmer Corp.). Maximal fluorescence is determined by the addition of 100 μ l of 10% Triton X-100 to lyse 1 ml of cells (*F*_{max}) and minimal fluorescence by the addition of 50 μ l of 1 M EGTA to chelate the calcium (*F*_{min}) (9). The pH of the lysed cells after addition of EGTA is 8.32. Corrections are made for the fluorescence in dimethylsulfoxide (DMSO)-treated hepatocytes, the quenching of fluorescence due to Triton X-100, and any solvent effects, e.g. alcohol. The cytosolic calcium content is determined by the equation $[Ca^{++}]_i = K_D(F - F_{min})/(F_{max} - F)$, where the dissociation constant (*K*_D) is 115 nM (9). Derivation of the equation has been previously outlined (10). Where indicated, 1,25(OH)₂D or 24,25 dihydroxyvitamin D (24,25(OH)₂D) (courtesy Hoffman LaRoche Inc., Nutley, NJ) or the calcium ionophore A23187 (Sigma Chemical Co., St. Louis, MO) are added to 1 ml of cells in 10 μ l 95% alcohol for 5 min. Controls are treated with 10 μ l 95% alcohol for 5 min. Probability of difference is determined by Student's paired *t* test.

Liver homogenate production of 25 OHD. The rat liver homogenate is prepared as we have previously described (4). Vitamin D-deficient rats are killed by exsanguination under light ether anesthesia and the livers removed and chilled immediately in ice-cold 0.25 M sucrose. Incubations include 5 ml of the liver homogenate, 2.5 ml of a buffer-cofactor solution, and 2.5 ml of a salt mixture. The latter consists of 5 mM MgCl₂ and 0.1 M KCl to give final concentrations of 1.25 mM MgCl₂ and 25 mM KCl in the incubation volume. The effect of the divalent ion calcium is studied by removal of equimolar amounts of MgCl₂ and substitution with CaCl₂. 2 pmol of [³H]vitamin D, 22 Ci/mmol (Amersham Corp., Arlington Heights, IL) are added to each flask in 25 μ l 95% alcohol. Incubations are carried out for 3 h and terminated as previously described (4). The identity of the [³H]25 OHD is confirmed by cochromatography with authentic 25 OHD (courtesy D. J. Babcock, Upjohn Co., Kalamazoo, MI) on Sepadex LH 20 and high-performance liquid chromatography (HPLC). The protein concentration is determined by the method of Lowry (11) and the data expressed as pmol [³H]25 OHD/g protein per 3 h. In those studies where EGTA is included in the homogenate mixture, the compound is added at the start of the 3-h incubation. Probability of difference is determined by analysis of variance.

Hepatocyte production of 25 OHD. Hepatocytes (1×10^7 viable cells) are suspended in 5 ml Biggers-Gwatkin-Judah (BGJ) culture medium containing albumin, 1 g/dl, and 5 ml of a salt-cofactor solution (4). The final incubation volume is 10 ml and contains 2 pmol [³H]vitamin D, 1,25(OH)₂D, 2 ng/ml, A23187, 3 μ M, and EGTA, 1.5 mM, are added to the appropriate flasks. Incubations are carried out for 90 min at 37°C and 60 oscillations per min, at which time cell viability is determined by trypan dye exclusion and the reactions terminated by addition of 25 ml MeOH and 12.5 ml CHCl₃. The identity of the [³H]25 OHD is confirmed as above and data expressed as picomoles [³H]25 OHD produced per 10^7 cells per 90 min. Probability of difference is determined by Student's *t* test.

Results

As seen in Fig. 1, the fluorescence emission spectrum of DMSO-treated hepatocytes at an excitation of 339 nm shows a maximum fluorescence between 450 and 470 nm and residual fluorescence at 495 nm. This autofluorescence is partly attributed to nucleotides within the cell. Quin 2-loaded hepatocytes demonstrate a

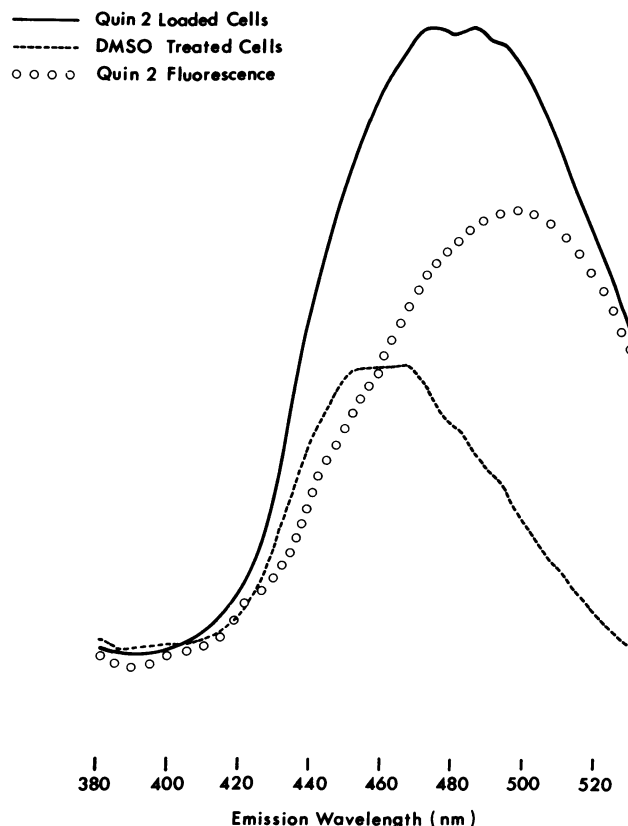


Figure 1. Emission spectrum of hepatocytes. Emission spectrum of 1 ml of Quin 2-loaded and 1 ml of DMSO-treated hepatocytes containing 10 μ l alcohol is measured between 380 and 530 nm. The emission spectrum for Quin 2 fluorescence is determined by subtracting the autofluorescence of the DMSO-treated cells from the fluorescence of Quin 2-loaded hepatocytes. The fluorescence of Quin 2 peaks between emission wavelengths of 490 and 500 nm.

maximal fluorescence between 470 and 490 nm. Subtraction of the autofluorescence of hepatocytes from the fluorescence of Quin 2-loaded cells reveals the emission spectrum for Quin 2, peaking between 490 and 500 nm (circles, Fig. 1). Treatment of Quin 2-loaded hepatocytes incubated in medium containing 0.5 mM calcium with 1,25(OH)₂D, 100 pg/ml, for 5 min increases the fluorescence of the cells (Fig. 2). This increment occurs gradually during the 5-min period while the fluorescence of alcohol-treated cells remains stable (Fig. 3). At the end of this 5-min period, fluorescence in the supernatant of control and 1,25(OH)₂D treated cells is identical, indicating that the increase in fluorescence is from within the cells and not due to leakage of Quin 2 into the medium. 1,25(OH)₂D, 100 pg/ml, increases hepatocyte cytosolic calcium levels by 24% (197 ± 18 vs. 244 ± 21 nM, $P < 0.001$, $n = 17$), while 1,25(OH)₂D, 1 ng/ml, increases levels by 39% (203 ± 12 vs. 282 ± 41 nM, $P < 0.04$, $n = 4$). At a concentration of 2 ng/ml, 1,25(OH)₂D treatment results in a 50% increase in cytosolic calcium levels (224 ± 26 vs. 337 ± 57 nM, $P < 0.04$, $n = 9$). In order to determine whether 1,25(OH)₂D is increasing cytosolic calcium by increasing membrane transport of calcium or by mobilizing intracellular calcium stores, 24 μ l of 1 M EGTA are added per milliliter of cells to chelate extracellular calcium prior to the addition of the vitamin D metabolite. In the absence of extracellular calcium, 1,25(OH)₂D, 2 ng/ml,

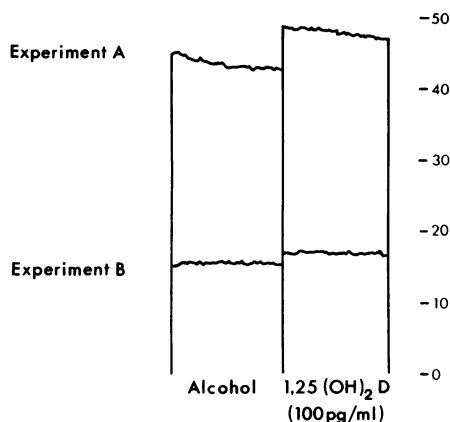


Figure 2. 1,25(OH)₂D increases cellular fluorescence. The effect of 1,25(OH)₂D, 100 pg/ml, on cellular fluorescence is measured in two cell preparations (experiments *A* and *B*) after 5 min of exposure to either 10 μ l alcohol or 1,25(OH)₂D in 10 μ l of alcohol. For demonstration purposes the fluorescence of each control preparation is arbitrarily set on the recorder. Readings are obtained at an excitation of 339 nm, emission of 495 nm with slit widths of 5 nm. Chart speed is 60 mm/min with a sensitivity range of 1,000 mV. 1,25(OH)₂D increases fluorescence by 4 U in experiment *A* and 2 U in *B*.

increases hepatocyte cytosolic calcium levels by 56% (204 ± 21 vs. 318 ± 65 nM, $P < 0.01$, $n = 5$). In contrast, 24,25(OH)₂D, 10 ng/ml, has no effect on cytosolic calcium levels (183 ± 35 vs. 188 ± 37 nM, $n = 6$). 1,25(OH)₂D and 24,25(OH)₂D have no effect on the autofluorescence of the DMSO-treated hepatocytes. At a concentration of 2 ng/ml, 1,25(OH)₂D decreases hepatocyte 25 OHD production by 32% (0.28 ± 0.02 vs. 0.19 ± 0.01 pmol [³H]25 OHD produced/ 10^7 cells per 90 min, $P < 0.01$, $n = 4$).

As seen in Fig. 4, addition of CaCl₂ to the liver homogenate inhibits [³H]25 OHD production. The control incubation containing 1.25 mM MgCl₂ and 25 mM KCl is represented by the bar at the far left. Decreasing the MgCl₂ concentration to 0.85 mM and increasing the KCl to 33 mM has no effect on [³H]25 OHD production. Although CaCl₂ at concentrations of 0.05 and 0.1 mM has no effect on [³H]25 OHD by the homogenates, synthesis in the presence of 0.2 mM CaCl₂, representing an ultrafilterable calcium concentration of 70 μ M (12), is decreased by 37%, $P < 0.02$, and is further decreased by greater concentrations of CaCl₂ (Fig. 4). EGTA alone has no effect on [³H]25 OHD synthesis. However, incubation of the liver homogenates

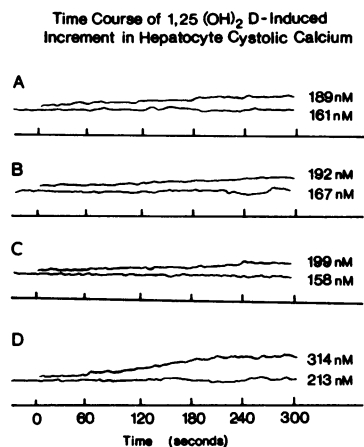


Figure 3. Time course of 1,25(OH)₂D-induced changes in cytosolic calcium. The effect of 1,25(OH)₂D on cellular fluorescence is measured over 5 min with constant stirring. Control cells were treated with ethanol, 10 μ l. The sensitivity range is 1,000 mV. Panels *A*, *B*, and *C* demonstrate the effect of 1,25(OH)₂D, 100 pg/ml, on cytosolic calcium levels, while in panel *D* the concentration of the vitamin D metabolite is 2 ng/ml.

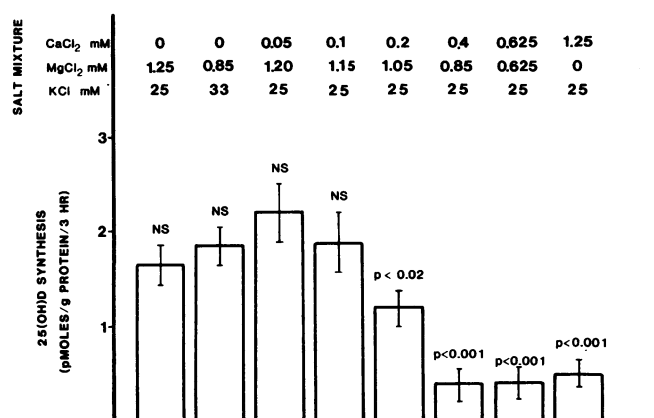


Figure 4. Calcium inhibits 25 OHD synthesis. [³H]25 OHD production is determined after 3 h of incubation of 2 pmol [³H]vitamin D in a 25% liver homogenate. The effect of CaCl₂ is determined by substitution of equimolar amounts of CaCl₂ for MgCl₂. Values represent the mean \pm SEM of 6–9 separate incubations for each salt mixture.

with 0.4 mM CaCl₂ and 5 mM EGTA to chelate the calcium prevents the calcium-induced inhibition (0.5 ± 0.1 vs. 1.3 ± 0.2 pmol [³H]25 OHD/g liver protein per 3 h, $P < 0.02$, $n = 6$), further documenting the role of the calcium ion as the inhibitory agent.

Since the minimum concentration of free calcium required to inhibit 25 OHD synthesis in liver homogenates is 70 μ M (350 times greater than cytosolic calcium), we examined the effect of increased cytosolic calcium on hepatocyte 25 OHD production. The calcium ionophore A23187, 3 μ M, increases hepatocyte cytosolic calcium by 52% (196 ± 11 vs. 298 ± 37 nM, $P < 0.05$, $n = 8$). Likewise, treatment with the ionophore decreases hepatocyte 25 OHD production (0.28 ± 0.02 vs. 0.20 ± 0.02 pmol [³H]25 OHD produced/ 10^7 cells per 90 min, $P < 0.05$, $n = 4$), an effect which is prevented by chelation of extracellular calcium with EGTA (0.28 ± 0.02 vs. 0.25 ± 0.02 pmol [³H]25 OHD produced/ 10^7 cells per 90 min).

Discussion

The results of this study clearly demonstrate that 1,25(OH)₂D is capable of increasing hepatocyte cytosolic calcium levels within 5 min by mobilizing intracellular calcium stores, that 1,25(OH)₂D inhibits hepatocyte 25 OHD production, that the calcium ion inhibits hepatic 25 OHD production in liver homogenates, and that increments in cytosolic calcium induced by the calcium ionophore are associated with decreased hepatocyte 25 OHD production. Alterations in cytosolic calcium levels have been shown to mediate a number of biochemical processes including parathyroid hormone release (13), insulin release (1, 2), prolactin release (10, 14), and vasopressin-induced increments in hepatocyte phosphorylase activity (15, 16). Cytosolic calcium levels in osteoblast-like cells are affected by alterations in extracellular calcium (17), while levels in UMR106 (18) and SaOS-2 (19) osteogenic sarcoma cells are increased by parathyroid hormone. Since the concentration of calcium required to inhibit 25 OHD production in liver homogenates is supraphysiologic, we have employed the calcium ionophore to increase cytosolic calcium levels to a degree comparable to 1,25(OH)₂D-treated cells. Ionophore-induced increments of cytosolic calcium within the physiologic range are associated with decreased 25

OHD production. The ionophore is used to demonstrate the relationship between changes in cytosolic calcium and 25 OHD synthesis rather than 1,25(OH)₂D, because in addition to increasing cytosolic calcium, the vitamin D metabolite has also been shown to be a direct noncompetitive inhibitor of the vitamin D 25 hydroxylase in liver homogenates (20). The observation that the ionophore effect is prevented by chelation of extracellular calcium demonstrates that the inhibitory action of A23187 is not a nonspecific toxic effect.

As an intracellular messenger, the free calcium ion concentration of the cytosol is critically important. The free calcium concentration is buffered by binding to proteins in the cytosol, exchange with calcium pools in the microsomes and mitochondria, and transport processes across the plasma membrane (21). Calcium enters the cell by a sodium channel and by a specific calcium channel which is affected by the calcium channel blockers. Cytosolic calcium levels are also regulated by active transport of calcium out of the cell via a specific ATP-dependent calcium pump and Na⁺/Ca⁺⁺ exchange (21). Hormone-induced increments in cytosolic free calcium appear to be due to either increased entry into the cell, decreased transport out of the cell, or mobilization of calcium from intracellular pools. In situations where cytosolic calcium increases in the absence of extracellular calcium, changes in phosphatidylinositol metabolism are believed to mediate the process (22). Vasopressin-induced breakdown of polyphosphoinositides in hepatocyte membranes is sufficiently rapid for it to have a primary role in generating messenger signals responsible for intracellular calcium mobilization (15, 16). In various cells, both inositol polyphosphates (16) and diacylglycerol (23) have been implicated in the generation of the calcium response.

The rapidity of the 1,25(OH)₂D-induced effect on hepatocyte cytosolic calcium levels is not unique for this metabolite. 1,25(OH)₂D enhances calcium transport in perfused duodena from normal chicks within 14 min (24). Epithelial cells from normal rat intestine demonstrate increased calcium uptake within 5 min of treatment with 1,25(OH)₂D in vitro (25), and calcium uptake of rat intestinal Golgi vesicles is enhanced within 15 min of 1,25(OH)₂D treatment in vivo (26). Therefore, the observations that 1,25(OH)₂D increases cytosolic calcium within 5 min raises the possibility that the mechanism is independent of genome activation, perhaps attributable to a direct interaction with membranes (24).

Other factors in addition to calcium and 1,25(OH)₂D have been reported to affect 25 OHD synthesis. Acute phenobarbital treatment (27) and an acid load (28) enhance 25 OHD production, whereas chronic phenobarbital treatment (7), cimetidine and isoniazid (29), and alcohol administration (30) inhibit production.

The vitamin D system is now recognized to play a role in the regulation of hematolymphopoietic tissue (31) as well as the maintenance of mineral and bone homeostasis. The current studies indicate that 1,25(OH)₂D increases hepatocyte cytosolic calcium levels by mobilizing intracellular pools, and that these increments may be involved in 1,25(OH)₂D-induced regulation of 25 OHD production (4–6). The vitamin D endocrine system is thus involved in both extracellular and intracellular calcium homeostasis.

Acknowledgments

This work was supported in part by National Institutes of Health grant AM35104.

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