

1995

Effects of vitamin D, dietary calcium and vitamin D restriction, pregnancy and lactation on gene expression of calcium transporting factors

Yingting Zhu
Iowa State University

Follow this and additional works at: <https://lib.dr.iastate.edu/rtd>

 Part of the [Animal Sciences Commons](#), [Nutrition Commons](#), [Physiology Commons](#), and the [Veterinary Physiology Commons](#)

Recommended Citation

Zhu, Yingting, "Effects of vitamin D, dietary calcium and vitamin D restriction, pregnancy and lactation on gene expression of calcium transporting factors " (1995). *Retrospective Theses and Dissertations*. 11105.
<https://lib.dr.iastate.edu/rtd/11105>

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

**Effects of vitamin D, dietary calcium and vitamin D restriction, pregnancy
and lactation on gene expression of calcium transporting factors**

by

Yingting Zhu

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment
of the Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

**Department: Veterinary Physiology and Pharmacology
Major: Physiology**

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

**Iowa State University
Ames, Iowa**

1995

UMI Number: 9611006

UMI Microform 9611006

Copyright 1996, by UMI Company. All rights reserved.

This microform edition is protected against unauthorized
copying under Title 17, United States Code.

UMI

300 North Zeeb Road
Ann Arbor, MI 48103

TABLE OF CONTENTS

1.	GENERAL INTRODUCTION	1
	Dissertation Organization	3
	Literature Review	4
	References	22
2.	REGULATION OF RAT INTESTINAL CaATPase AND CaBP mRNA BY 1,25-D₃ AND 1,25,28-D₂	42
	Abstract	42
	Introduction	43
	Materials and Methods	45
	Results	48
	Discussion	49
	Acknowledgment	51
	References	51
	Appendix with mean data tables	56
3.	EFFECT OF DIETARY CALCIUM AND VITAMIN RESTRICTION ON RAT INTESTINAL CaATPase AND CaBP mRNA EXPRESSION	69
	Abstract	69
	Introduction	70
	Materials and Methods	72
	Results	75
	Discussion	76
	Acknowledgment	77
	References	77
	Appendix with mean data tables	83
4.	PREGNANCY AND LACTATION INCREASE VITAMIN D-DEPENDENT INTESTINAL MEMBRANE CaATPase AND CaBP mRNA EXPRESSION	95
	Abstract	95
	Introduction	96
	Materials and methods	98
	Results	102
	Discussion	103

Acknowledgment	106
References	107
Appendix with mean data tables	115
5. GENERAL CONCLUSIONS	130

GENERAL INTRODUCTION

Details of mechanisms of intestinal calcium transport and its regulation by vitamin D, dietary calcium and vitamin D restriction, pregnancy and lactation are still not well understood. For example, several recent reports indicated that 1,25-dihydroxyvitamin D₃ [1,25-D₃] was not involved in ATP-dependent basolateral membrane calcium efflux (41) and did not enhance intestinal calcium binding protein [CaBP] and calcium ATPase [CaATPase] (25, 134, 138). These observations directly conflict with the classical theory that 1,25-D₃ binds to specific intracellular vitamin D receptors [VDR], to initiate transcription and translation of various products (60, 94, 102). Moreover, only a few reports on the regulation of gene expression of CaATPase by 1,25-D₃ in rat and chick intestine have been published (5, 21, 170). No reports on the regulation of gene expression of CaATPase by the vitamin D₂ analog, 1,25,28-D₂, have been published. The question of whether regulation of both CaATPase and CaBP by 1,25-D₃ and 1,25,28-D₂ were limiting factors in intestinal calcium transport was evaluated in the present study.

The question of whether dietary calcium is a regulator of gene

expression of calcium transporting factors is still a controversial subject which apparently needs further studies. Wasserman et al. (162) demonstrated restriction of dietary calcium upregulated intestinal CaATPase mRNA in chicks, directly contradictory to a report by Favus et al. (41) that ATP-dependent calcium uptake by isolated intestinal basolateral membrane vesicles was diminished when the vesicles were derived from rats fed calcium-deficient diet. Earlier reports indicated that dietary calcium restriction could induce CaBP mRNA expression (6, 7, 42, 97, 105, 113, 133), but recent papers demonstrated calcium restriction did not enhance CaBP mRNA expression (55, 136). Therefore, it is of great importance to evaluate whether the adaptation to calcium deficiencies involves inducing gene expression of intestinal CaATPase and CaBP.

The active transport of calcium by the intestinal tract is stimulated when large amounts of calcium are required during such physiological states as pregnancy and lactation (10, 47, 84, 140). Krisinger et al. (74) indicated that lactation increased intestinal CaBP mRNA expression. No reports have been published on the effects of pregnancy and lactation on intestinal CaATPase mRNA expression. Therefore, it is very important to determine which components of the intestinal active transport system are

activated.

This project was designed to investigate and compare the effects of 1,25-D₃ and 1,25,28-D₂ on mRNA expression of CaATPase and CaBP-9K in intestinal tissue, to determine whether adaptation to calcium deficiencies can induce gene expression of intestinal CaATPase and CaBP, and to study which components of intestinal active transport system are activated during late pregnancy and early lactation.

Dissertation Organization

This dissertation contains a general introduction which includes a literature review, three manuscripts and general discussion. The references cited in the general introduction are listed at the end of the chapter; references cited in each manuscript follow the manuscript. Figures and tables follow references [in the appendix]. The three manuscripts were written in the style of **Infection and Immunity**. The project was planned and executed, and the manuscripts written, primarily by the PhD candidate, Yingting Zhu, with the advice of the major professor, Dr. Jesse P. Goff and other committee members.

Literature Review

The mechanisms of intestinal calcium transport under physiological and pharmacological situations have been extensively studied in recent years. A transcellular and a paracellular pathway have been suggested for calcium transport in the intestine (17). The transcellular movement is dominant in the small intestine, whereas paracellular transport can occur anywhere throughout the intestine (107).

Intestinal calcium transport has the following characteristics:

1. When dietary calcium is low, active transport accounts for most of the calcium absorbed. This transcellular movement involves the diffusional entry of calcium into the intestinal mucosa cells across the brush border membrane, the diffusional translocation of calcium to the basolateral membrane and active extrusion of calcium by primary and secondary active calcium pump mechanisms.

2. Paracellular calcium transport is passive and down its concentration gradient. When the calcium concentration in the lumen of the intestine is high, the paracellular calcium transfer across the tight junction between enterocytes is predominant (37, 105, 161).

3. Calcium enclosed in membrane-bound vesicles can also be transported transcellularly (100).

The entry of calcium into cells can occur by simple diffusion across the membrane driven by the electrochemical gradient for Ca^{2+} , through voltage dependent or receptor-operated channels coupled to second messengers, or through the $\text{Ca}^{2+}/\text{Na}^{+}$ exchanger (2, 160).

Within cytosol, free calcium is closely regulated by the rate of entry and exit of calcium across the plasma membrane. The storage of calcium in the form of calcium binding proteins or in the intracellular organelles plays an important role in keeping intracellular ionized calcium levels low.

Because the free calcium level in cytosol is substantially lower than that of the extracellular milieu, the final resetting of intracellular calcium must

involve the extrusion of calcium out of cells through CaATPase and $\text{Na}^{+}/\text{Ca}^{2+}$ exchangers (112). Compared to CaATPase, the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger makes a relatively small contribution to calcium transport.

The $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger may be driven by $\text{Na}^{+}/\text{K}^{+}$ -ATPase (99).

It is well known that 1,25-dihydroxyvitamin D_3 [1,25- D_3] is a primary regulator of mineral homeostasis in intestine, bone and kidney (34).

This includes the induction of calcium binding proteins, basolateral

membrane CaATPase and alkaline phosphatase, an increase of calmodulin, and changes in membrane lipid composition (11, 51, 114, 167). The overall effect of vitamin D is to increase calcium absorption from the intestinal tract, to elevate calcium resorption from bones and to enhance the retention of calcium from the kidney. Parathyroid hormone stimulates calcium absorption by increasing renal enzyme 25-hydroxyvitamin D₃-1-hydroxylase (77). Estrogens may also enhance calcium absorption by indirectly increasing 1,25-D₃ in postmenopausal osteoporosis, pregnancy and lactation (45, 62, 73, 85). Other factors may also affect intestinal calcium homeostasis independent of vitamin D. For example, Halloran and DeLuca (82) reported that pregnancy and lactation increased intestinal calcium absorption even in vitamin D-deficient rats. Calcium must be released from the dietary components prior to its absorption, and transported across the intestinal enterocytes in a free ionized state (123). Gastric acid may enhance calcium solubilization from the diet, thus enhancing dietary calcium absorption (86). Exceptionally high dietary fibers may impair calcium absorption (117).

Vitamin D₃ is a steroid hormone. The metabolism and mechanism of action are similar to other steroid hormones. This vitamin can be derived

from the diet or synthesized from 7-dehydrocholesterol in the skin exposed to ultraviolet rays from the sun. Under ultraviolet light, the B-ring is opened to form previtamin D₃, which undergoes thermal equilibration to form vitamin D₃ (77). Vitamin D₃ and its metabolites circulate in the blood primarily bound to and in equilibrium with the vitamin D-binding globulin (154). The main function of the vitamin D-binding globulin is to protect and bind vitamin D, facilitate its solubility in the blood and act as a storage of vitamin D, increasing the half life in the body. In the mammalian liver, the vitamin is converted to an inactive precursor, 25-hydroxyvitamin D₃ [25-D₃], by 25-hydroxylase, a cytochrome P-450-like enzyme. The hydroxylation occurs in mitochondria and the microsomal fraction, requiring NADPH, O₂ and magnesium. In avian species, the intestine and kidney are also able to convert vitamin D₃ to 25-D₃ though this is probably of minor consequence (142). The rate of hydroxylation is limited by 25-D₃ feedback inhibition. In circulation 25-D₃ has a half life of about 2 to 3 weeks and can be stored in the liver. When required by the body, 25-D₃ is converted to the active form, 1,25-D₃, by the 1-hydroxylase in the renal tubular mitochondria of most mammalian and avian species (77). The conversion of 25-D₃ to 1,25-D₃ requires parathyroid hormone [PTH]. PTH

stimulation of 1-hydroxylase is mediated by cAMP, thus enhancing 1,25-D₃ production and intestinal calcium absorption (64, 77). PTH fails to increase intestinal calcium transport in vitamin D-deficient rats and humans (8, 46). It is clear that the stimulation of intestinal calcium transport by PTH is indirect, since it takes hours to develop. This is consistent with the time required for activation of the renal 1-hydroxylase to synthesize 1,25-D₃ and for its effects in the intestine (169). In the absence of PTH, little 1,25-D₃ can be formed and therefore, PTH is an important regulator of vitamin D. Conversely 1,25-D₃ feedback regulates the parathyroid gland and suppresses PTH secretion. The activity of renal 1-hydroxylase can be downregulated by increased plasma calcium and 1,25-dihydroxyvitamin D₃. Calcium itself has a slight inhibitory effect on conversion of 25-D₃ to 1,25-D₃. Calcium also inhibits the secretion of PTH, therefore preventing the conversion of 25-D₃ to 1,25-D₃. During pregnancy, the placenta plays a significant role in converting 25-D₃ to 1,25-D₃ (52, 132, 135, 164-166). Alternatively 25-D₃ can be converted by 24-hydroxylase to 24,25-dihydroxyvitamin D₃ [24,25-D₃] and other inactive metabolites when calcium and phosphate requirements are met. Metabolism of 1,25-D₃ occurs by side chain oxidation to form an inert 23-carbon acid, calcitric acid, in liver and

intestine or hydroxylation to less active 1,24,25-D₃, 1,25,26-D₃, 23-oxo-1,25-D₃, 23-oxo-1,26,26-D₃ or 1,25-D₃-23,26 lactone in target tissues such as intestine, kidney and cartilage (77). The metabolites are excreted in bile and can be reabsorbed in the small intestine, characteristic of an enterohepatic circulation.

1,25-D₃ targets its own intracellular vitamin D receptors [VDR], which belong to nuclear transcriptional factors of the steroid-thyroid receptor gene family (61, 111). The vitamin D receptor consists of a DNA binding domain at the amino terminus portion of the protein, a hinge region and a steroid binding domain at the carboxy terminus (28). The DNA binding domain contains several zinc fingers which are believed to interact directly with corresponding DNA. The steroid binding domain is the ligand binding domain. The receptor was found in cytosol and nucleus. A high-affinity, low capacity receptor for 1,25-D₃ with a sedimentation coefficient of 3.2 S to 3.5 S has been identified from cytosolic homogenates of intestine in chicks, rats, human and other species, with highest affinity for 1,25-D₃ and a thousand-fold lower affinity for 25-D₃ and other metabolites (18, 59, 109, 147). Two subspecies of 1,25-D₃ receptors of 58 kDa and 60 kDa for avian species and two of 52 kDa and 55 kDa for mammalian species have

been characterized (30, 110). These were confirmed by in vitro translation of 1,25-D₃ receptor mRNA (87). The 1,25-D₃ receptors are distributed in many tissues such as intestine, kidney, bone, brain, breast, cartilage, pancreas, parathyroid, pituitary, testis, thymus, thyroid and skin tissues. No VDR has been found in neural cells or skeletal muscle (28). The cDNA of VDR for rat, chick and human have been cloned (90), making it possible to study gene regulation of VDR.

The action of 1,25-D₃ on the VDR is similar to other steroid hormones on their receptors. 1,25-D₃ can passively enter its target cells, bind with cytosolic unoccupied receptors to be ferried into the nucleus or diffuse into the nucleus and directly binds to nuclear unoccupied receptors. This binding evokes conformational changes that increase receptor affinity to specific vitamin D response elements [VDREs] located in the promoter region of vitamin D-dependent genes (58, 103, 148). Interaction between ligand-receptor complex and VDREs modulates transcription of mRNA biology of the cell and thus mediate the vitamin D response. VDREs have been identified for the calbindin-9K, rat osteocalcin, mouse osteopontin and human osteocalcin genes (28).

It is very clear that vitamin D exerts its effects on a wide range of

issues. Similar to other steroids, the vitamin regulates cellular functions by changing gene transcription, mRNA production and stability and protein synthesis (154). VDR is a substrate for protein kinase A and other protein kinases (28). The phosphorylation of VDR by protein kinase A or other protein kinases is required for its activation and transcriptional activity, because the phosphorylation of VDR correlates with its ability to activate transcription. Interestingly, the phosphorylation was found to occur only at serine residues (28). The reason for the phosphorylation at these particular sites is unknown. VDR enhances transcellular calcium transport in the intestine and kidney, and stimulates bone calcium resorption (35, 75, 89, 102, 116). The hormone-receptor complex, with other transcriptional factors, may increase transcription activities of RNA polymerase II (77). Despite the efforts of research scientists for several decades, not all the mechanisms of transport nor the controlling factors involving the calcium transport process have been found (4, 102).

The stimulation of calcium uptake in several cell types by 1,25-D₃ has been reported. This response can be blocked by cycloheximide and/or actinomycin D, providing direct evidence of a transcription related effect (3, 70, 168). The effects of 1,25-D₃ on calcium uptake among different cell

types also differ in magnitude and in time course (3, 149, 168).

1,25-D₃ can also elicit a nongenomic rapid response. The response includes transcaltachia and opening of voltage-gated channels. Intracellular calcium, cAMP, cGMP and protein kinase C may be involved in these processes (29, 31, 32, 163). Nemere and Norman (100) demonstrated that 1,25-D₃ induced a rapid increase in calcium transport in a vascularly perfused duodenal preparation from normal chicks. deBoland and Norman (33) believe that the response is the result of activation of basolateral calcium channels mediated by protein kinase A [PKA] and protein kinase C [PKC]. Although the vitamin can activate non-genomic inositol phospholipid mechanism to initiate rapid tissue responsiveness, research scientists have been making more efforts to illustrate the genomic regulatory mechanisms initiated by vitamin D.

The vitamin D dependency of the nonsaturable components [passive transport] of calcium absorption is still controversial. Wasserman et al. (163) and Dostal et al. (37) indicated both saturable and nonsaturable processes are enhanced by vitamin D. However, not all scientists agree (163).

The vitamin D receptor undergoes autoregulation (27, 81, 104). The

action of 1,25-D₃ could cause transient hypercalcemia, thus suppressing PTH secretion. The decreased PTH levels can in turn diminish the synthesis of 1,25-D₃ (77). Conflicting results have been reported on whether vitamin D increases vitamin D receptor mRNA (40, 68, 91, 131). Since injecting rats with 1,25-D₃ increases VDR concentration, if VDR mRNA does not increase, this would imply a change in VDR probably at the post-transcriptional level, rather than the transcriptional level.

Although analogs and metabolites of vitamin D have been tested for their calcitropic activity, there are few reports of the effects of these compounds on gene expression (155). It was reported that a dose as high as 800 ng of 1,25,28-trihydroxyvitamin D₂ [1,25,28-D₂] had no effects on rat intestinal calcium transport, but a dose as low as 250 ng upregulated intestinal CaBP mRNA (155). Whether the analogs of 1,25-D₃, such as 1,25,28-D₂, regulate intestinal CaATPase mRNA has not been reported. No changes in plasma calcium have been observed in vitamin D-deficient rats injected with 7 ug of 1,25,28-D₂ per day for three months (Horst and Reinhardt, unpublished).

Calcium binding protein is the most extensively studied vitamin D-dependent gene product. In mammals, CaBP-9K has a molecular size of

approximately 9 kDa, is present in the intestine, and mouse kidney, whereas CaBP-28K has a molecular size of 28 kDa, is primarily found in the mammalian kidney, brain, pancreas and avian intestine(26). These proteins can be found in calcium-transporting tissues such as intestine, kidney, bones and some non-calcium transporting tissues such as brain and pancreas (38, 69, 76, 137, 141, 156). Some of these 1,25-D₃-dependent calcium binding proteins are believed to stimulate intestinal calcium transport (54, 63, 97, 120). 1,25-D₃ markedly increases the vitamin D-dependent CaBPs and their mRNA in mammals, chick intestine, kidney and skin (54, 63, 77, 102, 108, 125). However, some observations are inconsistent with the theory of direct involvement of CaBP in calcium translocation. For example, a recent report indicated that in vitamin D-repleted rats, duodenal calcium transport was enhanced by a single injection of calcitriol, whereas CaBP-9K remained unchanged (25). Interestingly, some 1,25-D₃ non-responsive organs such as heart and lung have significant levels of CaBPs and their mRNA (152, 153). No obligatory relationships have been found between 1,25-D₃ effects and CaBP induction (152). Multiple factors such as calcium, phosphate, glucocorticoids, sex steroids and age are believed to participate in CaBP regulation (1, 20, 26, 54, 63, 90, 102, 143). For example, no active calcium

transport is reported in the intestine of newborn rats (39, 106), with undetectable levels of CaBP (106, 144). Even large dose of 1,25-D₃ cannot induce CaBP in newborn rats, but can induce both calcium transport and CaBP in older rats (13, 144). This is probably due to lack of receptors for 1,25-D₃ in the newborn rats (57) or possibly due to lack of response from undeveloped kidneys. Both the active transport of calcium and calcium binding protein levels reach a peak at about 30 days of age in rats, and then gradually decrease (106). A previous report has suggested that intestinal CaBP-9K mRNA was upregulated only in lactation and not in pregnancy (74). Bruns et al. (19) suggested that active transport in the duodenum increased during lactation. The enhanced calcium transport cannot be entirely explained by the increase in 1,25-D₃ concentration, because Halloran and DeLuca (56) found significantly increased calcium transport ratio in vitamin D-deficient lactating rats that lacked detectable levels of circulating 1,25-D₃. A close relationship between 1,25-D₃, intestinal CaBP and calcium transport has been observed in which the induction of CaBP coincides with increased calcium transport and this is induced by vitamin D, but not calcium (158, 159). The important functions of CaBPs include 1,25-D₃-induced calcium transport and buffering of the intracellular calcium pool (15, 16,

63). Intestinal vitamin D-dependent calcium binding protein [CaBP-9K] was also reported to stimulate vesicular calcium transport (151). The structural analysis of CaBPs showed that CaBP-28K contains six highly conserved EF-hand domains and binds 4 calcium per molecule, whereas CaBP-9K binds 2 calcium per molecule and contains only 2 EF-hand domains (77, 80). The structures were initially described by Hunziker (69) as "loop-helix-loop structures that bind calcium to various oxygen-containing residues within the loop region". CaBPs may activate and bind to CaATPase (43, 44, 71, 96, 119, 151, 157). However, some observations were inconsistent with the theory of direct involvement of vitamin D in calcium translocation (36, 49). Therefore, it is necessary to reevaluate the transcriptional regulation of CaBP-9K by 1,25-D₃.

Most of the initial work on the characterization, isolation, purification and reconstitution of the plasma membrane calcium pump has been performed on the enzyme present in human red blood cells (128). Later, the enzyme was found in the plasma membrane of all other types of cells and was proposed as a ubiquitous enzyme (22). The CaATPase is a calcium pump which actually transports calcium out of the cytosol in order to maintain the concentration gradient of calcium across plasma membrane. The

typical CaATPase of the plasma membrane belongs to the P-type ATPase. A characteristic property of these enzymes is the formation of a covalently phosphorylated, obligatory intermediate; hence, the name P-type ATPase arises from the transfer of a phosphate of ATP to a specific aspartate residue at the catalytic site of the peptide (128). Recently a vitamin D-dependent plasma membrane calcium ATPase [PMCA1] gene has been cloned (126, 145), making it possible to study calcium homeostasis more thoroughly. Schatzmann (22, 124) first observed Ca²⁺-pump activity of the plasma membrane of red blood cells, and later Carafoli (22) reported that all eukaryotic cells studied have this enzymatic activity. Several authors proposed that plasma membrane CaATPase has 2 ATP-binding domains with different ATP affinity (97, 127). Although the mechanism is not clear, the higher affinity domain is suggested as the catalytic site; the lower one as a regulator (124). PMCA has a molecular weight of 130-140 kDa and transports one calcium per ATP. The calcium transport cycle is triggered by the ATP-dependent phosphorylation of an aspartyl residue, and the activity is augmented by the ATP-dependent phosphorylation of serine and threonine residues catalyzed by protein kinase A and C (163). At least four genes coding for plasma membrane CaATPases [PMCA1, PMCA2, PMCA3

and PMCA4] have been found (24, 66). In rat cDNA libraries, all four isoforms of PMCA and their mRNA have been identified (53, 66, 126). PMCA4 was also discovered in human cells (130). The isoform variability of PMCA appears to be generated by alternative splicing of single gene primary transcripts (53, 126, 128, 129). The reasons for the diversity of the enzyme are still a matter under investigation. Strehler (128) reported that highly conserved sequences are likely to represent domains essential for the basic catalytic and transport function and may also reflect specific constraints imposed on structural elements of the enzyme. In contrast, highly divergent sequences probably specify isoform-specific regulatory and functional specifications of the enzyme that are adapted to the physiological needs of the tissue in which the corresponding enzyme is expressed (128). PMCA1 was found to be ubiquitous, whereas other isoforms were expressed in a tissue-dependent manner (53). In the duodenum, PMCA1 is predominant and regulated by vitamin D (5, 21, 170). The other isoforms of CaATPase have not been reported to be regulated by vitamin D. In the intestine 1,25-D₃ enhances calcium transport, in part, by stimulating epithelial cell PMCA1 mRNA and CaATPase activity (48, 75, 79, 87, 89, 91, 170). An increase of CaATPase activity by 1,25-D₃ has also been reported in cartilage cells (83)

and vascular smooth muscle cells (72) but not in circulating mononuclear cells (93). There seems to be a correlation between the cellular location and magnitude of activation of CaATPase, the sites and induction of calcium transport in intestine and kidney (54, 63). In rats, the calcium ATPase activity parallels the distribution of calcium absorption activity, with the highest activity in duodenum. This activity is reduced in vitamin D deficiency (48, 150). Calmodulin stimulates CaATPase to form a phosphorylated intermediate (146). This converts the enzyme into a high calcium affinity state in which the affinity for calcium increases 30-fold and the affinity for ATP increases 100-fold (121, 123). Phosphorylated metabolites of phosphatidylinositol have been found to be activators of CaATPase and phosphorylation by the cAMP-dependent protein kinase could also activate CaATPase (23). Phorbol esters and diacylglycerol are activators of calcium extrusion from intact cells (78, 118). A rat placental CaATPase mRNA was shown to be upregulated during late pregnancy and early lactation (50). However, little is known of changes in rat intestinal CaATPase mRNA expression during late pregnancy and early lactation. The rate of calcium absorption in the aging rat decreases. This is partially due to decreased mRNA and protein synthesis, decreased activity of CaATPase and

CaBP, thus decreased ATP-dependent calcium uptake by basolateral membrane of enterocytes (5).

It has been demonstrated that 1,25-D₃ regulates PMCA1 in rat and chick intestine (5, 21, 170). However, several recent biochemical studies indicated that 1,25-D₃ did not enhance the synthesis of intestinal PMCA1 (134, 138), and did not enhance ATP-dependent basolateral membrane calcium efflux (41). Therefore, it is important to re-evaluate gene regulation of CaATPase and CaBP by 1,25-D₃.

Calcium or phosphorus deficiencies have been reported to induce CaATPase gene expression and also to enhance calcium pump activity (21, 162). This contradicted a report by Favus et al. (41) which indicated that ATP-dependent calcium uptake by isolated intestinal basolateral membrane vesicles was diminished when the vesicles were derived from rats fed a calcium-deficient diet. Whether calcium restriction or calcium itself induces upregulation of CaATPase and CaBP-9K mRNA is still a controversial subject, which apparently needs further studies. For example, the expression of CaBP-9K dropped after 24 hours in a calcium-free medium with 1 mM EGTA and an increase of media calcium concentration to 1.2 mM induced a 6- to 10-fold increase of CaBP-9K mRNA which can be blocked

by actinomycin D in duodenal cell culture (12). The increase of duodenal calcium transport in chicks by 1,25-D₃ was found to be dependent on the presence of media calcium, since it was abolished by prior addition of EGTA and was restored upon addition of calcium (31). The reduced dietary calcium was reported to increase intestinal CaBP mRNA, but at the same time to diminish VDR mRNA despite increased circulating 1,25-D₃ (93). No difference in the steady state of intestinal CaBP mRNA levels was found between rats fed low or normal calcium diet (136). In vitamin D-deficient status, dietary calcium manipulation did not affect either intestinal CaBP or its mRNA in chicks, but a single injection of 1,25-D₃-induced equivalent duodenal CaBP and corresponding mRNA (55). However, earlier reports suggested that the adaptation to calcium-deficient diet was associated with an increased CaBP and its mRNA (6, 7, 42, 97, 105, 113, 133).

In summary, whether transcriptional regulation of both CaATPase and CaBP are limiting factors in intestinal calcium transport and whether the adaptation to calcium and/or vitamin D deficiencies affects gene expression of intestinal CaATPase and CaBP, and which components of active transport system are activated during late pregnancy and early lactation, are still not well understood. The present investigation attempted to answer these

questions.

References

- 1. Abe, H., Amano, O., Yamakuni, T., Kuwano, R., Takahashi, Y. & Kondo, H. 1991.** Transient expression of a calcium-binding protein (spot 35-calbindin) and its mRNA in the immature pituicytes of embryonic rats. *Cell Tissue Res.* 266:325-330.
- 2. Adams, D. J., Barakeh, J., Laskey, R. and Van Breemen, C. 1989.** Ion channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB J.* 3:2389-2400.
- 3. Akerstrom, V. L. & Walters, M. R. 1992.** Physiological effects of 1,25-dihydroxyvitamin D3 in the TM4 Sertoli cell line. *Am. J. Physiol.* 262:E884-E890.
- 4. Antrobus, S. A., Hunziker, W. & Walters, M. R. 1990.** Examination of interaction of calbindin-D28K with cytosolic proteins in the rat kidney and brain using the gel overlap technique. *Int. Symp. calcium binding proteins.* 7:168A.
- 5. Armbrecht, H. J., Boltz, M. A. & Wongsurawat, N. 1994.** Expression of plasma membrane calcium pump mRNA in rat intestine: effect of age and 1,25-dihydroxyvitamin D. *Biochim. Biophys. Acta.* 1195:110-114.
- 6. Bar, A. & Wasseman, R. H. 1973.** Control of calcium absorption and intestinal calcium binding protein synthesis. *Biochem. Biophys. Res. Commun.* 54:191-196.
- 7. Bar, A. & Hurwitz, S. 1979.** The interaction between dietary calcium and gonadal hormones in their effect on plasma calcium, bone, 25-hydroxycholecalciferol-1-hydroxylase, and duodenal calcium-binding protein, measured by a radioimmunoassay in chicks. *Endocrinology.* 104:1455-1460.

- 8. Baerg, R. D., Kimberg, D. V. & Gershon, E. 1970.** Effect of renal insufficiency on the active transport of calcium by the small intestine. *J. Clin. Invest.* 49:1288-1300.
- 9. Bikle, D. D., Nemanic, M. K., Whitney, J. O. & Elias, P. W. 1986.** Neonatal human foreskin keratinocytes produce 1,25-dihydroxyvitamin D₃. *Biochemistry.* 25:1545-1548.
- 10. Boass, A., Lovdal, J. A. & Toverud, S. U. 1992.** Pregnancy- and lactation-induced changes in active intestinal calcium transport in rats. *Am. J. Physiol.* 262:G127-G134.
- 11. Brasitus, T. A., Dudeja, P. K., Eby, B. & Lau, K. 1986.** Correction by 1,25-dihydrocholecalciferol of enterocyte brush border membranes in vitamin D-deprived rats. *J. Biol. Chem.* 261: 16404-16409.
- 12. Brehier, A. & Thomasset, M. 1990.** Stimulation of calbindin-D9K (CaBP9K) gene expression by calcium and 1,25-dihydroxyvitamin D₃ in fetal duodenal organ culture. *Endocrinology.* 127:580-587.
- 13. Bronner, F., Lipton, J., Pansu, D., Buckley, M., Singh, R., and Miller, A. 1982.** Molecular and transport effects of 1,25-dihydroxyvitamin D₃ in rat duodenum. *Fed. Proc.* 41:61-65.
- 14. Bronner, F. 1987.** In: *Physiology of the gastrointestinal tract* (2nd ed.), edited by L. R. Johnson. New York: Raven. p 1419-1435.
- 15. Bronner, F. & Stein, W. D. 1988.** CaBPr facilitates intracellular diffusion for Ca pumping in distal convoluted tubule. *Am. J. Physiol.* 255:F558-F562.
- 16. Bronner, F. 1990.** Transcellular calcium transport. In: Bronner F. (ed) *intracellular calcium metabolism*. Alan R. Liss, Inc., New York, p 415.
- 17. Bronner, F. 1992.** Current concepts of calcium absorption: An overview. *J. Nutr.* 122:641-643.

- 18. Brumbaugh, P. F. & Haussler, M. R.** 1973. 1,25-dihydroxyvitamin D₃ receptor; competitive binding of vitamin D analogs. *Life Sci.* 13:1713-1746.
- 19. Bruns, M. E., Boass & Tuverud, S. U.** 1987. Regulation by dietary calcium of vitamin D-dependent calcium-binding protein and active calcium transport in the small intestine of lactating rats. *Am. J. Endocrinol.* 121:278-283.
- 20. Bruns, M. E., Overpeck, J. G., Smith, G. C., Hirsch, G. N., Mills, S. E. & Bruns, D. E.** 1988. Vitamin D-dependent calcium binding protein in rat uterus: differential effects of estrogen, tamoxifen, progesterone, and pregnancy on accumulation and cellular localization. *Endocrinology.* 122:2371-2378.
- 21. Cai, Q., Chandler, J. S., Wasserman, R. H., Kumar, R., Penniston, J. T.** 1993. Vitamin D and adaptation to dietary calcium and phosphate deficiencies increase the intestinal plasma membrane calcium pump gene expression. *Proc. Natl. Acad. Sci.* 90:1345-1349.
- 22. Carafoli, E.** 1987. Intracellular calcium homeostasis. *Annu. Rev. Biochem.* 56:395-433.
- 23. Carafoli, E.** 1988. Membrane transport of calcium: an overview. *Methods Enzymol.* 157:3-11.
- 24. Carafoli, E.** 1992. The Ca²⁺ pump of plasma membrane. *J. Biol. Chem.* 267:2115-2118.
- 25. Chabanis, S., Hanrotel, C., Duchambon, P., Banide, H., Kubrusly, M., Aymard, P., Lacour, B. & Druke, T.** 1994. Duodenal calcium binding protein and active transport in rats: are they functionally related? *Nephrol. Dial. Transplant.* 9:1402-1407.
- 26. Christakos, S., Gabrielides, C. & Rhoten W. B.** 1989. Vitamin D-dependent calcium binding proteins: chemistry, distribution, functional considerations, and molecular biology. *Endocrine Rev.* 10:3-16.

- 27. Costa, E. M., Hirst, M. A. & Feldman, D. 1985.** Regulation of 1,25-dihydroxyvitamin D₃ receptors by vitamin D analogs in cultured mammalian cells. *Endocrinology*. 117:2203-2210.
- 28. Darwish, H. M., Burmester, J. K., Moss, V. & DeLuca 1993.** Transcriptional activity by the 1,25-dihydroxyvitamin D₃ receptor is mediated by phosphorylation. *Biochim Biophys. Acta*. 1167:29-36.
- 29. deBoland, A. R. & Norman, A. W. 1990.** Evidence for involvement of protein kinase C and cyclic adenosine 3',5' monophosphate-dependent protein kinase in the 1,25-dihydroxyvitamin D₃-mediated rapid stimulation of intestinal calcium transport (transcaltachia). *Endocrinology*. 127:39-45.
- 30. Dame, M. C., Pierce, E. A. & DeLuca, H. F. 1985.** Identification of the porcine intestinal 1,25-dihydroxyvitamin D₃ receptor on sodium dodecyl sulphate/polyacryamide gels by renaturation and immunoblotting. *Proc. Natl. Acad. Sci. USA* 82:7825-7829.
- 31. deBoland, A. R. & Norman, A. W. 1990.** Influx of extracellular calcium mediates 1,25-dihydroxyvitamin D₃-dependent transcaltachia (the rapid stimulation of duodenal Ca²⁺ transport). *Endocrinology*. 127:2475-2480.
- 32. deBoland, A. R., Nemere, I. & Norman, A. W. 1990.** Ca²⁺ channel agonist bay K8644 mimics 1,25-dihydroxyvitamin D₃ rapid enhancement of Ca²⁺ transport in chick perfused duodenum. *Biochem. Biophys. Res. Commun*. 166:217-222.
- 33. de Boland, A. R. & Norma, A. W. 1990.** Evidence for involvement of protein kinase C and cyclic adenosine 3',5' monophosphate-dependent protein kinase in the 1,25-dihydroxyvitamin D₃-mediated rapid stimulation of intestinal calcium transport (Transcaltachia). *Endocrinology*. 127:39-45.
- 34. DeLuca, H. F. 1988.** The vitamin story: a collaborative effort of basic science and clinical medicine. *FASEB J*. 2:224-236.

- 35. Deluca, H. F., Krisinger, J. & Darwish, H. 1990. The vitamin D system: 1990 Kidney Int. 38:S2-S8.**
- 36. Donahue, H. J., Borke, J. L., Penniston, J. T. & Kumar, R. 1988. Effect of purified 28-KDa calcium binding protein (CaBP) on calmodulin-stimulated human erythrocyte (Ca^{2+} - Mg^{2+}) ATPase kinetics. J. Bone Mineral Res. 3:S51.**
- 37. Dostal, L. A. & Toverud, S. U. 1984. Effect of vitamin D on duodanal calcium absorption in vivo during development. Am. J. Physiol. 246:G528-G534.**
- 38. Desplan, C., Thomasset, M. & Moukhtar, M. 1983. Synthesis, molecular cloning, and restriction analysis of DNA complementary to vitamin D-dependent calcium-binding protein mRNA from rat duodenum. 258: 2762-2765.**
- 39. Dostal, L. A. & Toverud, S. U. 1984. Effect of vitamin D₃ on duodenal calcium absorption in vivo during early development. Am. J. Physiol. 246:G528-G534.**
- 40. Favus, M. J., Mangelsdorf, D. J., Tembe, V., Coe, B. J. & Haussler, M. R. 1988. Evidence for in vivo upregulation of the intestinal vitamin D receptor during dietary calcium restriction in the rat. J. Clin. Invest. 82:218-224.**
- 41. Favus, M. J., Tenbe, V., Ambrosic, K. A. & Nellans, H. N. 1989. Effects of 1,25-dihydroxyvitamin D₃ on enterocyte basolateral membrane calcium transport in rats. Am. J. Physiol. 256:G613-G617.**
- 42. Fox, J., Pickard, D. W., Care, A. D. & Murray, T. M. 1978. Effect of low phosphorus diet on intestinal calcium absorption and the concentration of calcium-binding protein in intact and parathyroidectomized pigs. Endocrinology. 78:379-387.**
- 43. Freund, T. S. 1982. In Norman, A. W., Schaefer, K., Herrath, Dv. & Grigoleit, H. G. (eds) Vitamin D: chemical, biochemical and clinical endocrinology of calcium metabolism. Walter de Gruyter, Berlin, p 249.**

44. Freund, T. S. & Christakos, S. 1985. Enzyme modification by renal calcium binding proteins. In Norman, A. W., Schaefer, K., Grigoleit, H. G. & Herrath, D. V. (eds) Vitamin D. A chemical, biochemical and clinical update. Walter de Gruyter & Co., Berlin, p 369.
45. Gallagher, J. C., Riggs, B. L. & Deluca, H. F. 1980. Effect of estrogen on calcium absorption and serum vitamin D metabolites in postmenopausal osteoporosis. *J. Clin. Endocrinol. Metab.* 51:1359-1364.
46. Garabedian, M., Tanaka, Y., Holick, M. F. 1974. Response of intestinal calcium transport and bone calcium mobilization to 1,25-dihydroxyvitamin D₃ in thyroparathyroidectomized rats. *Endocrinology.* 94:1022-1027.
47. Gertner, J. M., Coustan, D. R., Kliger, A. S., Mallette, L. E., Ravin, N. & Broadus, A. E. 1986. Pregnancy as state of physiologic absorptive hypercalciuria. *Am. J. Med.* 81:451-456.
48. Ghijsen, W. E. & Van Os C. H. 1982. 1,25-dihydroxyvitamin D₃ regulates ATP-dependent calcium transport in basolateral plasma membranes of rat enterocytes. *Biochim. Biophys. Acta.* 689:170-172.
49. Ghijsen, W. E., Van Os C. H., Heizmann, C. W. & Murer, H. 1986. Regulation of duodenal Ca²⁺ pump by calmodulin and vitamin D-dependent Ca²⁺-binding protein. *Am. J. Physiol.* 251:G223-G229.
50. Glazier, J. D., Atkinson, D. E., Thornburg, K. L., Sharpe, P. T., Edwards, D., Boyd, R. D. H. & Sibley, C. P. 1992. Gestational changes in Ca²⁺ transport across rat placenta and mRNA for calbindin-9K and Ca⁽²⁺⁾-ATPase. *Am. J. Physiol.* 262:R930-R935.
51. Goodman, D. B. P., Haussler, M. R. & Rasmussen, H. 1972. Vitamin D₃ induced alteration of microvillar membrane lipid composition. *Biochem. Biophys. Res. Commun.* 46:80-86.
52. Gray, T. K., Lester, G. E. & Lorenc, R. S. 1979. Evidence for extrarenal 1-hydroxylation of 25-hydroxyvitamin D₃ in pregnancy. *Science.* 204:1311-1313.

- 53. Greeb, J. and Shull, G. E. 1989.** Molecular cloning of a third isoform of the calmodulin-sensitive plasma membrane Ca^{2+} transporting ATPase that is expressed predominantly in brain and skeletal muscle. *J. Biol. Chem.* 264:18569-18576.
- 54. Gross, M. & Kumar, R. 1990.** Physiology and biochemistry of vitamin D-dependent calcium binding proteins. *Am. J. Physiol.* 259:F195-F209.
- 55. Hall, A. K. & Norman, A. W. 1990.** Regulation of calbindin-D28K gene expression in the chick intestine: effects of serum calcium status and 1,25-dihydroxyvitamin D₃. *J. Bone Miner. Res.* 5:331-336.
- 56. Halloran, B. P. & DeLuca, H. F. 1980.** Calcium transport in small intestine during pregnancy and lactation. *Am. J. Physiol.* 239:E64-E68.
- 57. Halloran, B. P. & DeLuca, H. F. 1981.** Appearance of the intestinal cytosolic receptor for 1,25-dihydroxyvitamin D₃ during neonatal development of the rat. *J. Biol. Chem.* 256:7338-7342.
- 58. Hannah, S. S. & Norman, A. W. 1994.** 1,25-(OH)₂ vitamin D₃-regulated expression of eukaryotic genome. *Nutr. Rev.* 52:376-382.
- 59. Haussler, M. R., Donaldson, C. A. & Kelly, M. A. et al 1985.** Function and mechanism of action of the 1,25-dihydroxyvitamin D₃ receptor. In Norman, A. W., Schaefer, K., Grigoleit, H-G, von Herrath, D. (eds): *Vitamin D: Chemistry, biochemical and clinical update*. Berlin, Walter de Gruyter, p 83-92.
- 60. Haussler, M. R. 1986.** Vitamin D receptors: nature and function. *Annu. Rev. Nutr.* 6:527-562.
- 61. Haussler, M. R., Mangelsdorf, D. J., Komm, B. S., Terpening, C. M., Yamaoka, K., Allegretto, E. A., Baker, A. R., Shine, J., McDonnell, D. P., Hughes, M., Weigel, N. L., O'Malley, B. W. & Pike, J. W. 1988.** Molecular biology of the vitamin D hormone. *Recent Prog. Horm. Res.* 44:263-305.

- 62. Heaney, R. P. & Skillman, T. G.** 1971. Calcium metabolism in normal human pregnancy. *J. Clin. Endocrinol. Metab.* 33:661-669.
- 63. Heimann, C. W. & Hunziker, H.** 1990. Intracellular calcium-binding molecules. In: Bronner F (ed) *Intracellular calcium regulation*. Alan. R. Liss, Inc., New York, p 211.
- 64. Horiuchi, N., Suda, T., Takahashi, H., Shimazawa, E. & Ogata, E.** 1977. In vivo evidence for the intermediary role of 3',5'-cyclic AMP in parathyroid hormone-induced 1, 25-dihydroxyvitamin D₃ synthesis in rats. *Endocrinology.* 101:969-974.
- 65. Howard, A., Legon, S., and Walters, J. R. F.** 1992. Plasma membrane calcium pump expression in human placenta and small intestine. *Biochem. Biophys. Res. Commun.* 183:499-505.
- 66. Howard, A., Legon, S. & Walters, J. F.** 1993. Human and rat intestinal plasma membrane calcium pump isoforms. *Am. J. Physiol.* G917-G925.
- 67. Howard, G. A., Turner, R. T., Sherrard, D. J. & Baylink, D. J.** 1981. Human bone cells in culture metabolize 25-hydroxyvitamin D₃ to 1,25-dihydroxyvitamin D₃ and 24,25-dihydroxyvitamin D₃. *J. Biol. Chem.* 256:7738-7740.
- 68. Huang, Y. C., Lee, S., Stolz, R. & Gabrielides, C.** 1989. Effect of hormone and development on the expression of the rat 1,25-dihydroxyvitamin D₃ receptor gene. Comparison with calbindin gene expression. 264:17454-17461.
- 69. Hunziker, W.** 1986. The 28-kDa vitamin D-dependent calcium-binding protein has a six domain structure. *Proc. Natl. Acad. Sci. USA* 83:7578-7582.
- 70. Inoue, T. & Kawashima H.** 1988. 1,25-dihydroxyvitamin D₃ stimulates ⁴⁵Ca²⁺-uptake by cultured vascular smooth muscle cells derived from rat aorta. *Biochem. Biophys. Res. Commun.* 152:1388-1394.

- 71. James, P. Vorherr, T., Thulin, E., Forsen, S. & Carafoli, E.** 1991. Identification and primary structure of a calbindin 9K binding domain in the plasma membrane Ca^{2+} pump. *FEBS Lett* 278:155-159.
- 72. Kawashima, H.** 1988. 1,25-dihydroxyvitamin D_3 stimulates Ca-ATPase in a vascular smooth muscle cell line. *Biochem .Biophys. Res. Commun.* 150:1138-1143.
- 73. Kostial K., Gruden, N. & Durakovic, A.** 1969. Intestinal absorption of ^{47}Ca and ^{85}Sr in lactating rats. *Calcif. Tissue Res.* 4:113-119.
- 74. Krisinger, J., Dann, J. L., Jeung, E. B. & Leung, P. C. K.** 1992. Calbindin-D9K gene expression during pregnancy and lactation in the rat. *Mol. and Cell. Endocrinol.* 88:119-128.
- 75. Kumar, R.** 1986. The metabolism and mechanism of action of 1,25-dihydroxyvitamin D_3 . *Kid. Int.* 30:793-803.
- 76. Kumar, R., Wieben, E. & Beecher, S. J.** 1989. The molecular cloning of the complementary deoxyribonucleic acid for bovine vitamin D-dependent calcium-binding protein: structure of the full length protein and evidence for homologies with other calcium-binding proteins of the troponin-C superfamily of proteins. *Mol. Endocrinol.* 3:427-432.
- 77. Kumar, R.** 1991. Vitamin D and calcium transport. *Kid. Int.* 40:1177-1189.
- 78. Lagast, H., Lew, P. D. & Waldvogel, F. A.** 1984. Adenosine triphosphate-dependent calcium pump in the plasma membrane of guinea pig and human neutrophils. *J. Clin. Invest.* 73:107-115.
- 79. Lane, S. M. & Lawson, D. E. M.** 1978. Differentiation of the changes in alkaline phosphatase from calcium ion-activated adenosine triphosphatase activities associated with increased calcium absorption in chick intestine. *Biochem. J.* 174:1067-1070 .

- 80. Leathers, V. L., Linse, S., Forsn S. & Norman , A. W. 1990.** Calbindin-D28K, a 1,25-dihydroxyvitamin D₃-induced calcium-binding protein, binds five or six Ca²⁺ ions with high affinity. *J. Biol. Chem.* 265:9838-9841.
- 81. Lee, Y., Inaba, M., DeLuca, H. F. & Melon, W. S. 1989.** Immunological identification of 1,25-dihydroxyvitamin D₃ receptors in human promyelocytic leukemic cells (HL-60) during homologous regulation. *J. Biol. Chem.* 264:13701-13705.
- 82. Lee, D. B. N., Hardwick, L. L, Hu, M. S. and Jamgotchian, N. 1990.** Vitamin D-independent regulation of calcium and phosphate absorption. *Miner. Electr. Metab.* 16:167-173.
- 83. Lidor, C. & Edelstein, S. 1987.** Calcitriol increases Ca²⁺-ATPase activity. *Biochem. Biophys. Res. Commun.* 144:713-717.
- 84. Lobaugh, B., Boass, A., Lester, G. E. & Toverud, S. U. 1990.** Regulation of serum 1,25-dihydroxyvitamin D₃ in lactating rats. *Am. J. Physiol.* 259:E665-671.
- 85. Lund, B. & Selnes, A. 1979.** Plasma 1,25-dihydroxyvitamin D levels in pregnancy and lactation. *Acta. Endocrinol.* 92:330-335.
- 86. Mahoney, A. W., Holbrook, B. S. & Hendrick, D. G. 1975.** Effects of calcium solubility or absorption by rats with induced achlorhydria. *Nutr. Metab.* 18:310-317.
- 87. Mangelsdorf, D. J., Pike, J. W. & Haussler, M. R. 1987.** Avian and mammalian receptors for 1,25-dihydroxyvitamin D₃: in vitro translation to characterize size and hormone dependent regulation. *Proc. Natl. Acad. Sci. USA* 84:354-358.
- 88. Martin, D. L., Melancon, M. J. & Deluca, H. F. 1969.** Vitamin D stimulated, calcium-dependent adenosine triphosphatase from brush borders of rat small intestine. *Biochem. Biophys. Res. Commun.* 35:819-823.

- 89. Marx, S. J., Liberman, U. A. & Eil, C. 1983. Calciferols: actions and deficiencies of action. *Vitam. Horm.* 40:235-308.**
- 90. Mathieu, C. L., Burnett, S. H., Mills S. E., Overpeck, J. G., Bruns, D. E. & Bruns, M. E. 1989. Gestational changes in calbindin-D9K in rat uterus, yolk sac, and placenta: implications for maternal-fetal calcium transport and uterine muscle function. *Proc. Natl. Acad. Sci. USA* 86:3433-3437.**
- 91. McDonnell, D. P., Mangelsdorf, D. J., Pike, J. W., Haussler, M. R. & O'Malley, B. W. 1987. Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. *Science.* 235:1214-1217.**
- 92. Melancon, M. J. & DeLuca, H. F. 1970. Vitamin D stimulation of calcium-dependent adenosine triphosphatase in chick intestinal brush borders. *Biochemistry.* 9:1658-1664.**
- 93. Meyer, J., Fullmer, C. S., Wasserman, R. H., Komm, B. S. & Haussler, M. R. 1992. Dietary restriction of calcium, phosphorus and vitamin D elicits differential regulation of the mRNAs for avian intestinal calbindin-D28K and the 1,25-dihydroxyvitamin D receptor. *J. Bone Miner. Res.* 7:441-448.**
- 94. Minghetti, P. P. & Norman, A. W. 1988. 1,25-dihydroxyvitamin D3 receptors: gene regulation and genetic circuitry. *FASEB J.* 2:3043-3053.**
- 95. Morgan, D. W., Welton, A. F., Heick, A. E. & Christakos, S. 1986. Specific in vitro activation of Ca,Mg-ATPase by vitamin-dependent rat renal calcium binding protein (calbindin D28K). *Biochem. Biophys. Res. Commun.* 138:547-553.**
- 96. Morimoto, S., Birge, S. J., Shen, V. Avioli, L. V. 1985. (Ca²⁺ + Mg²⁺)-ATPase activity in plasma membrane of circulating mononuclear cells. Lack of direct effect of vitamin D. *J. Biol. Chem.* 260:14953-14957.**

- 97. Morrissey, R. L. & Wasserman, R. H.** 1971. Calcium absorption and calcium-binding protein in chicks on different calcium and phosphate intakes. *Am. J. Physiol.* 220:1509-1515.
- 98. Muallen, S. & Karlish, S. J.** 1980. Regulatory interaction between calmodulin and ATP on the red cell Ca^{2+} pump. *Biochim. Biophys. Acta.* 597:631-636.
- 99. Nellans, H. N. & Popovitch, J. R.** 1984. Role of sodium in intestinal calcium transport. In: *Epithelial calcium and phosphate transport: molecular and cellular aspects.* (Bronner, F. & Peterlik M. eds), p 301-306, AlanR. Liss, New York, NY.
- 100. Nemere, I. & Norman, A. W.** 1990. Transcaltachia, vesicular calcium transport, and microtubule-associated calbindin-D28K: Emerging views of 1,25-dihydroxyvitamin D_3 -mediated intestinal calcium absorption. *Mineral and Electro. Metabolism* 16:109-114.
- 101. Nemere, I. & Norman, A. W.** 1991. Steroid hormone actions at the plasma membrane: induced calcium uptake and exocytotic events. *Mol. Cell Endocrinol.* 80:C165-C169.
- 102. Norman, A. W., Roth, J. & Orci, L.** 1982. The vitamin D endocrine system: steroid metabolism, hormone receptors and biological response (calcium binding protein). *Endocr. Rev.* 3:331-366.
- 103. Ozono, K., Sone, T. & Pike, J. W.** 1991. The genomic mechanism of action of 1,25-dihydroxyvitamin D_3 . *J. Bone Mineral Res.* 6:1021-1027.
- 104. Pan, L. C. & Price, P. A.** 1987. Ligand-dependent regulation of the 1,25-dihydroxyvitamin D_3 receptor in rat osteosarcoma cells. *J. Biol. Chem.* 262:4670-4675.
- 105. Pansu, D., Ballaton, C. & Bronner F.** 1981. Effect of Ca intake on saturable and nonsaturable components of duodenal Ca transport. *Am. J. Physiol.* G32-G37.

- 106. Pansu, D., Bellaton, C. & Bronner, F. 1983. Developmental changes in the mechanisms of duodenal calcium transport in the rat. Am. J. Physiol. 244:G20-G26.**
- 107. Pansu, D., Bellaton, C., Roche, C. & Bronner, F. 1983. Duodenal and ileal calcium absorption in the rat and effects of vitamin D. Am. J. Physiol. 244:G695-G700.**
- 108. Pavlovitch, J. H., Didierjean, L., Rizk, M., Balsan, S. & Saurat, J. H. 1983. Skin calcium-binding protein: distribution in other tissues. Am. J. Physiol. 244:C50-C57.**
- 109. Pike, J. W. 1985. Intracellular receptors mediate the biological action of 1,25-dihydroxyvitamin D₃. Nutr. Rev. 43:161-168.**
- 110. Pike, J. W. 1985. New insights into mediators of vitamin D₃ action. in vitamin D: Chemical, biological and clinical update. Norman, A. W., Schaefer, K., Grigoleit, H-G. & von Herrath, D., Eds., Walter de Gruyter, Berlin, p 97.**
- 111. Pike, J. W. 1991. Vitamin D₃ receptors: Structure and function in transcription. Annu. Rev. Nutr. 11:189-216.**
- 112. Pietrobon, D., Divirgilio, F. & Pozzan, T. 1990. Structural and functional aspects of calcium homeostasis in eukaryotic cells. Eur. J. Biochem. 193:599-622.**
- 113. Rader, J. I., Baylink, D. J., Hughes, M., Safilian, E. F. & Haussler, M. R. 1979. Calcium and phosphorus deficiency in rats: effects on PTH and 1,25-dihydroxyvitamin D₃. Am. J. Physiol. 236:E118-E122.**
- 114. Rasmussen, H., Matsumoto, T., Fontaine, O. & Goodman, D. B. P. 1982. Role of changes in membrane lipid structure in the action of 1,25-dihydroxyvitamin D₃. Fed. Proc. 41:72-77.**
- 115. Reichel, H., Koeffler, H. P. & Barbers, R. et al 1985. 1,25-dihydroxyvitamin D₃ and the hematopoietic system. In Norman, A. W.,**

Schaefer, K., Grigoleit, H-G. & von Herrath, D. (eds): vitamin D: chemical, biological & clinical update. Berlin, Walter de Gruyter, pp 167-176.

116. Reichel, H., Koeffler, H. P. & Norman, A. W. 1989. The role of vitamin D endocrine system in health and disease. *N. Engl. J. Med.* 320:980-991.

117. Reinhold, J. G., Faradji, B., Abadi, P. & Ismail-Beigi. F. 1976. Decreased absorption of calcium, magnesium, zinc and phosphorus by human due to increased fiber and phosphorus consumption as wheat bread. *J. Nutr.* 106:493-503.

118. Rickard, J. E. & Sheterline, P. 1987. Evidence that phorbol ester interferes with stimulated Ca^{2+} redistribution by activating Ca^{2+} efflux in neutrophil leucocytes. *Biochem. J.* 231, 623-628.

119. Reisner, P. D. & Christakos, S. & Vanaman, T. C. 1992. In vitro enzyme activation with calbindin-D28K, the vitamin D-dependent 28 kDa calcium binding protein. *FEBS Lett.* 297:127-131.

120. Roche, C., Bellaton, C., Pansu, D., Miller, A., & Bronner, F. 1986. Localization of vitamin D-dependent active calcium transport and relationship to CaBP. *Am. J. Physiol.* 251:G314-G320.

121. Rossi, J. P., Rega, A. F. & Garrahan, P. J. 1985. Compound 48/80 and calmodulin modify the interaction of ATP with the $(\text{Ca}^{2+}+\text{Mg}^{2+})$ -ATPase of red cell membranes. *Biochim. Biophys. Acta.* 816:379-386.

122. Schacter, D., Dowdle, E. & Schenker, H. 1960. Active transport of calcium by the small intestine of the rats. *Am. J. Physiol.* 198:263-268.

123. Scharff, O. & Foder, B. 1982. Rate constants for calmodulin binding to Ca^{2+} -ATPase in erythrocyte membranes. *Biochim. Biophys. Acta.* 691:133-143.

124. Schatzmann, H. J. 1989. The calcium pump of the surface membrane and the sarcoplasmic reticulum. *Annu. Rev. Physiol.* 51:473-485.

- 125. Schelling, C. P., Didierjean L., Rizk, M., Pavlovitch, J. H., Takagi, T. & Heizmann, C. W. 1987. Calcium-binding proteins in rat skin. FEBS Lett. 214:21-27.**
- 126. Shull, G. E. & Greeb, J. 1988. Molecular cloning of two isoforms of the plasma membrane Ca^{2+} -transporting ATPase from rat brain. J. Biol. Chem. 263:8646-8657.**
- 127. Stieger, J. & Luterbacher, S. 1981. Some properties of purified ($\text{Ca}^{2+}+\text{Mg}^{2+}$)-ATPase from human red cell membranes. Biochim. Biophys. Acta. 641:270-275.**
- 128. Strehler, E. (1991) Recent advances in the molecular characterization of plasma membrane Ca^{2+} pumps. J. Membr. Biol. 1991. 120:1-15.**
- 129. Strehler, E. E., Strhler-Page, M. A., Vogel, G. & Carafoli, E. 1989. mRNAs for plasma membrane calcium pump isoforms differing in their regulatory domain are generated by alternative splicing that involves two internal donor sites in a single exon. Proc. Natl. Acad. Sci. USA 86:6908-6912.**
- 130. Strehler, E. E., James, P., Fischer, R., Heim, R., Vorherr, T., Filoteo, A. G., Penniston, J. T. & Carafoli, E. 1990. Peptide sequence analysis and molecular cloning reveal two calcium pump isoforms in the human erythrocyte membrane. J. Biol. Chem. 265:2835-2842.**
- 131. Strom, M., Sandgren, M. E., Brown T. A. & DeLuca, H. F. 1989. 1,25-dihydroxyvitamin D₃ upregulates the 1,25-dihydroxyvitamin D₃ receptor in vivo. Proc. Natl. Acad. Sci. USA 86:9770-9773.**
- 132. Surago, S., Horiuchi, N. & Takahashi, N. 1979. The site of 1,25-dihydroxyvitamin D₃ production in pregnancy. Biochem. Biophys. Res. Commun. 90:948-955.**
- 133. Swaminathan, R., Sommerville, B. A. & Care, A. D. 1977. The effect of dietary calcium on the activity of 25-hydroxycholecalciferol-1-**

hydroxylase and Ca absorption in vitamin D-replete chicks. *Br. J. Nutr.* 38, 47-54.

134. Takito, J., Shinki, T., Tanaka, H. & Suda, T. 1992. Mechanism of regulation of calcium pumping activity in chick intestine. *Am. J. Physiol.* 262:G797-G805.

135. Tanaka, Y., Halloran, B., Schnoes, H. K. & Deluca, H. F. 1979. In vitro production of 1,25-dihydroxyvitamin D₃ by rat placental tissue. *Proc. Natl. Acad. Sci. USA* 76:5033-5035.

136. Theofan, G., King, M. W., Hall, A. K. & Norman, A. W. 1987. Expression of calbindin-D28K mRNA as a function of altered serum calcium and phosphorus levels in vitamin D-replete chick intestine. *Mol. Cell Endocrinol.* 54:135-140.

137. Thomasset, M., Parkes, C. O. & Cuisinier-Gleizes, P. 1982. Rat calcium binding proteins: distribution, development, and vitamin D dependence. *Am. J. Physiol.* 243:E483-E488.

138. Timmermans, J. A., Kaune, R., Bindels, R. J. & van Os C. H. 1991. Quantification of Ca⁽²⁺⁾-ATPases in porcine duodenum. Effect of 1,25-dihydroxyvitamin D₃ deficiency. *Biochim. Biophys. Acta.* 1065:177-184.

139. Tornquist, K. & Tashjian, A. H. 1989. Dual actions of 1,25 dihydroxycholecalciferol on intracellular Ca²⁺ in GH4C1 cells: evidence for effects on voltage-operated Ca²⁺ channels and Na⁺/Ca²⁺ exchanger. *Endocrinology.* 124:2765-2776.

140. Toverud, S. U., Boass, A., Haussler, M. R. & Pike, J. W. 1983. Circulating levels and function of 1,25-dihydroxyvitamin D₃ in lactation. *J. Steroid Biochem.* 19:505-510.

141. Tsarbopoulos, A., Gross, M., Kumar, R. & Jardine, I. 1989. Rapid identification of calbindin-D28K cyanogenbromide peptide fragments by plasma absorption mass spectrometry. *Biomed. Environ. Mass Spectrom.* 18:387-393.

- 142. Tucker, G. III, Gagnon, R. E. & Haussler M. R. 1973.** Vitamin D₃-25-hydroxylase. Tissue occurrence and apparent lack of regulation. *Arch. Biochem. Biophys.* 155:47-57.
- 143. Varghese, S., Lee, S., Huang, Y-C & Christakos, S. 1988.** Analysis of rat vitamin D-dependent calbindin-D28K gene expression. *J. Biol. Chem.* 263:9776-9784.
- 144. Ueng, T. H., Golub, E. E. & Bronner, F. 1979.** The effect of age and 1,25-dihydroxyvitamin D₃ treatment on the intestinal calcium-binding protein of suckling rats. *Arch. Biochem. Biophys.* 196:624-630.
- 145. Verma, A. K., Filoteo, A. G., Stanford, D. R., Wieben, E. D., Penniston, J. T., Strehler, E. E., Fischer, R., Heim, R., Vogel, G., Mathew, Strehler-Page M. A., James, P., Vorherr, T., Krebs, J. & Carafoli, E. 1988.** Complete primary structure of a human plasma membrane Ca²⁺ pump. *J. Biol. Chem.* 263:14152-14159.
- 146. Wajsman, R., Walters, J. R. F. & Weiser, M. M. 1988.** Identification and isolation of the phosphorylated intermediate of the calcium pump in rat enterocyte basolateral membranes. *biochem. J.* 256:593-598.
- 147. Walters M. R., Hunziker, W., Norman, A. W. 1980.** Unoccupied 1,25-dihydroxyvitamin D₃ receptors; nuclear/cytosol ratio depends on ionic strength. *J. Biol. Chem.* 255:6799-6805.
- 148. Walters, M. R. 1985.** Steroid hormone receptors and the nucleus. *Endocr. Rev.* 6:512-543.
- 149. Walters, M. R., Ilenchuk, T. T. & Claycomb, W. C. 1987.** 1,25-dihydroxyvitamin D₃ stimulates Ca²⁺ uptake by cultured adult rat ventricular cardiac muscle cells. *J. Biol. Chem.* 262:2536-2541.
- 150. Walters, J. R. F. & Weiser, M. M. 1987.** Calcium transport by rat duodenal villus and crypt basolateral membranes. *Am. J. Physiol.* 252:G170-G177.

- 151. Walters, J. R. F.** 1989. Calbindin-D9K stimulate calcium pump in rat enterocyte basolateral membranes. *Am. J. Physiol.* 256:G124-G128.
- 152. Walters, M. R., Bruns, M. E., Carter, R. M. & Riggle, P. C.** 1991. Vitamin D-independence of small calcium binding proteins in nonclassical target tissues. *Am. J. Physiol.* 260:E794-E800.
- 153. Walters, M. R. & Hunziker, W.** 1991. Northern and PCR analysis of CaBP-D28K Expression. In Norman, A. W., Bouillon, R. & Thomasset, M. (eds) *Vitamin D: gene regulation, structure-function analysis and clinical applications.* Walter de Gruyter & Co., New York, p 619.
- 154. Walters, M. R.** 1992. Newly identified actions of the vitamin D endocrine system. *Endocrine Reviews.* 13:719-764.
- 155. Wang, Y. Z., Li, H., Bruns, M. E., Uskokovic, M., Truitt, G. A., Horst, R., Reinhardt, T. & Christakos, S.** 1993. Effect of 1,25,28-trihydroxyvitamin D₂ and 1,24,25-trihydroxyvitamin D₃ on intestinal calbindin-D9K mRNA and protein: is there a correlation with intestinal calcium transport? *J. Bone Miner. Res.* 8:1483-1490.
- 156. Wasserman, R. H., Corradino, R. A. & Taylor, A. N.** 1968. Vitamin D-dependent calcium-binding protein. Purification and some properties. *J. Biol. Chem.* 243:3978-3986.
- 157. Wasserman, R. H.** 1980. In: Siegel, F. L., Carofili, E., Kretsinger, R. H., Malennan, D. H. & Wasserman, R. H. (eds) *Calcium binding protein: structure and function.* Elsevier-North Holland, Inc., New York, p 357.
- 158. Wasserman, R. H.** 1981. Intestinal absorption of calcium and phosphorus. *Fed. Proc.* 40:68-72.
- 159. Wasserman, R. H., Fullmer, C. S. & Shimura, F.** 1984. Calcium absorption and the molecular effects of vitamin D₃, in *Vitamin D: basic and clinical aspects*, edited by Kumar, R., Boston, Martinus Nijhoff. p 233-302.

- 160. Wasserman, R. T.** 1988. Cellular calcium and phosphate transport in health and disease, p 3-13, Alan R. Liss, Inc.
- 161. Wasserman, R. H., Chandler, J. S., Meyer, S. A., Smith, C. A., Brindak, M. E., Fullmer, C. S., Penniston, J. T. & Kumer, R.** 1992. Intestinal calcium transport and calcium extrusion processes at basolateral membrane. *J. Nutr.* 122:662-671.
- 162. Wasserman, R. H., Smith, C. A., Brindak, M. E., Talamoni, N., Fullmer, C. S., Penniston, J. T. & Kumar, R.** 1992. Vitamin D and mineral deficiencies increase the plasma membrane calcium of chick intestine. *Gastroenterology.* 102:886-894.
- 163. Wasserman, R. H. & Fullmer, C. S.** 1995. Vitamin D and intestinal calcium transport: Facts, speculations and hypotheses. *J. Nutr.* 125:1971S-1979S.
- 164. Weisman, Y., Vargas, A., Duckett, G.** 1978. Synthesis of 1,25-dihydroxyvitamin D in the nephrectomized pregnant rats. *Endocrinology.* 103:1992-1996.
- 165. Weisman, Y., Harell, A. & Edelstein, S.** 1979. 1,25-dihydroxyvitamin D₃ and 24,24-dihydroxyvitamin D₃ in vitro synthesis by human decidua and placenta. *Nature.* 281:317-319.
- 166. Whitsett, J. A., Ho, M. & Tsang, R. C.** 1981. Synthesis of 1,25-Dihydroxyvitamin D₃ by human placenta in vitro. *J. Clin. Endocrinol. Metab.* 53:484-488.
- 167. Max, E. E., Goodman, D. B. P. & Rasmussen, H.** 1978. Purification and characterization of chick intestine brush border membrane; effects of 1-hydroxyvitamin D₃ treatment. *Biochim. Biophys. Acta.* 511:224-239.
- 168. Xue, H., McCarron, D. A. & Bukoski, R. D.** 1991. Vitamin D₃-induced ⁴⁵Ca²⁺ uptake in vascular myocytes from spontaneously hypertensive and normotensive rats. *Life Sci.* 49:651-659.

- 169. Yamamoto, M. & Kawanobe, Y. 1984. Vitamin D deficiency and renal calcium transport in the rat. *J. Clin. Invest.* 74:507-513.**
- 170. Zelinski, J. M., Sykes, D. E. & Weiser, M. M. 1991. The effect of vitamin D on rat intestinal plasma membrane Ca-pump mRNA. *Biochem Biophys. Res. Commun.* 179:749-755.**

REGULATION OF RAT INTESTINAL CaATPase AND CaBP mRNA BY 1,25- DIHYDROXYVITAMIN D₃ AND 1,25,28- TRIHYDROXYVITAMIN D₂

A paper to be submitted to the J. of Bone and Miner. Res.

Yingting Zhu, Jesse P. Goff, Timothy A. Reinhardt and Ronald L. Horst

Abstract

1,25-dihydroxyvitamin D₃ is a primary regulator of intestinal active calcium transport. The purpose of this study was to determine the changes in intestinal vitamin D-dependent plasma membrane calcium ATPase 1 [PMCA1] and calcium binding protein-9K [CaBP-9K] mRNA induced by a single injection of 1,25-dihydroxyvitamin D₃ or an analog of 1,25-dihydroxyvitamin D₃ that is considered non-hypercalcemic, 1,25,28-trihydroxyvitamin D₂, in vitamin D-deficient rats. The results of Northern blotting of duodenal tissues indicated that a single injection of 20 ng of 1,25-dihydroxyvitamin D₃ per rat doubled intestinal PMCA1 at 4, 8, and 12 hours after injection, and CaBP-9K mRNA was increased at 8, 12, and 24 hours after injection. No increase in PMCA1 and CaBP-9K mRNA

was observed in duodenum of rats receiving an injection of 1 ug of 1,25,28-trihydroxyvitamin D₂, which also had no effect on plasma calcium concentration. However, the mRNA of PMCA1 and CaBP-9K could be increased by a larger dose of 1,25,28-trihydroxyvitamin D₂ [20 µg], which was accompanied by an increase in plasma calcium. These data suggest that upregulation of PMCA1 and CaBP-9K mRNA may be critical to active calcium transport in the intestine.

Introduction

Active calcium transport in the intestine involves three steps: 1. calcium diffusion into brush border membrane of the enterocyte down its concentration gradient; 2. translocation of free or bound calcium to the basolateral membrane; and 3. active extrusion of calcium across basolateral membrane against a concentration gradient (3, 31). Inside cells, calcium binding protein serves as an intracellular calcium "carrier" (31). At the basolateral membrane, calcium ATPase [CaATPase] pumps calcium into the extracellular fluid against a concentration gradient.

The active metabolite of vitamin D, 1,25-dihydroxyvitamin D₃ [1,25-D₃] is a primary regulator of calcium and phosphorus transport in

the intestine. As with other steroid hormones, 1,25-D₃ binds to specific intracellular receptors, i.e., vitamin D receptors [VDR], to initiate transcription and translation of various gene products (14, 15, 23, 24). Two gene products thought to be involved in calcium transport are CaATPase and CaBP (11, 17-22, 24, 35). However, some observations are inconsistent with the theory of direct involvement of vitamin D in calcium translocation. For example, a recent report indicated that, in vitamin D-repleted rats, duodenal calcium transport was enhanced by a single injection of calcitriol, whereas CaBP-9K stayed essentially unchanged (5). This conflicts with reports of studies that increased calcium transport coincided with increased CaBP (6, 8, 29, 30). It has been demonstrated that 1,25-D₃ upregulates PMCA1 mRNA in rat and chick intestine (1, 4, 35), but recent reports reveal that 1,25-D₃ does not enhance the synthesis of intestinal PMCA (27, 28), and did not affect ATP-dependent basolateral membrane calcium efflux (10). Therefore, it is important to re-evaluate the transcriptional regulation of PMCA1 and CaBP-9K by 1,25-D₃.

Although analogs and metabolites of vitamin D have been tested for their calcitropic activity, few reports have documented the effects of these compounds on gene expression (34). It was reported that doses as high as

800 ng of 1,25,28-trihydroxyvitamin D₂ [1,25,28-D₂] did not affect rat intestinal calcium transport, but doses as little as 250 ng of 1,25,28-D₂ upregulated intestinal CaBP mRNA (34). No changes in plasma calcium have been found in vitamin D-deficient rats injected with 7 ug of 1,25,28-D₂ per day for three months (Horst and Reinhardt, unpublished). These data suggest that upregulation of CaBP was not the only limiting factor in intestinal calcium transport in these experiments. In this study, we determined whether upregulation of both CaATPase and CaBP-9K were limiting factors in intestinal calcium transport by comparing the effects of 1,25-D₃ and 1,25,28-D₂ [at doses that did and did not cause an increase in plasma calcium] on mRNA expression of calcium ATPase and CaBP-9K in intestinal tissue of rats.

Materials and Methods

Animals: Three week-old male Holtzman Sprague-Dawley weaning rats were fed a synthetic calcium deficient [-Ca], vitamin D deficient [-D] diet [0.002% calcium, 0.41 % phosphate] (Tekland, Madison, WI) for three weeks to induce vitamin D deficiency and hypocalcemia. Treatment groups consisted of 4-5 rats.

In trial 1, rats were injected i. p. with 20 ng of 1,25-D₃ or 20 ug of 1,25,28-D₂ at 4, 8, 12 and 24 h prior to sacrifice.

In trial 2, rats were injected i. p. with 20 ng of 1,25-D₃ or 1 ug of 1,25,28-D₂ at 4, 8, 12, 16, 24 and 36 hours prior to sacrifice. Control rats received no injection. Rats were killed by inhalation of CO₂-O₂ [50:50] followed by exsanguination. Blood samples were collected into heparinized tubes. The proximal 15 cm of duodenum mucosa was collected from each rat and frozen for later mRNA analysis.

Northern blotting: Plasma CaATPase and CaBP-9K mRNA

levels were determined by Northern blotting. Total RNA was isolated by TRIZOL and chloroform, precipitated and washed by isopropanol and ethanol (GIBCO, BRL, Gaithersburg, MD). PolyA-containing RNA was selected via oligo dT cellulose affinity chromatography (2). RNA was fractionated on 1.2% formaldehyde agarose gel and transferred to nylon membranes (MSI Micro Separations Inc., Westboro, MA). The RNA on the membranes was cross-linked by Stratalinker 1800 (Stratagene, La Jolla, CA). A 3.4 kb cDNA from rat brain plasma membrane calcium ATPase [PMCA1], kindly supplied by Dr Gary Shull, University of Cincinnati, OH, was used to hybridize the membranes for analysis of CaATPase mRNA.

The cDNA for CaBP-9K mRNA assay was the gift of Dr. M. Elizabeth Bruns, University of Virginia Medical School, Charlottesville, VA.

Membranes were prehybridized at 42^o C for 4-6 hours in a prehybridization buffer containing 50% formamide, 5X Denhardt's reagent, 0.1% sodium dodecyl sulfate [SDS] and 100 ug/ml denatured fish sperm DNA. Fresh prehybridization buffer and 250 ug tRNA was used for the overnight membrane hybridization with 5 x 10⁶ cpm ³²P-labeled cDNA probe/ml prehybridization buffer. The membranes were washed twice in 2X SSPE/0.1% SDS buffer [SSPE is NaCl, NaP₂PO₄, EDTA mixture], twice in 0.1% SSPE/0.1% SDS, each for ten minutes.

Quantitative analysis of mRNA was done by Scanalytics AMBIS imaging systems (Division of CSPI, San Diego, CA). A 2.1 kb chick β-actin cDNA probe was obtained by digestion of pBR322 with Hind III (7) and used as a control for estimation of mRNA integrity and RNA sample loading difference among various preparations.

Plasma calcium concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer Corp., Norwalk, CT).

Analysis of variance was used to determine if differences existed among CaATPase, CaBP-9K mRNA levels and plasma calcium

concentrations at each time point. When ANOVA suggested differences existed, Dunnett multiple comparisons were used to test the significance between mRNA levels at time 0 with all other time points.

Results

Intestinal PMCA1 mRNA concentration increased 1.89-, 1.94- and 1.77-fold at 4, 8, 12 hours after injection of 20 ng 1,25-D₃ [$p < 0.01$ at 4 and 8 hours and $p < 0.05$ at 12 hours respectively] (Figure 1, 3 and Table 1) when compared with time 0. PMCA1 mRNA concentration increased 2.1-fold at 8 hours after treatment with 20 ug of 1,25,28-D₂ [$p < 0.01$] (Figure 2, 3 and Table 1). A significant increase of CaBP mRNA was also found at 8, 12 and 24 hours after injection of 20 ng of 1,25-D₃ (Figure 4, 6 and Table 2) and at 4, 8, 12 and 24 hours after injection of 20 ug of 1,25,28-D₂ (Figure 5, 6 and Table 2). However, injection of 1 ug of 1,25,28-D₂ failed to increase either PMCA1 or CaBP-9K mRNA levels (Figure 7 and 8).

Interestingly, significantly higher plasma calcium concentrations were observed at 4, 8 and 12 hours after injection of 20 ug of 1,25,28-D₂ (Figure 9, Table 3). No significant increase of plasma calcium occurred

after injection of 20 ng of 1,25-D₃ at any time point when compared to time 0 (Figure 10, Table 3).

Discussion

Active calcium transport across the intestine involves calcium entry across the brush border of enterocytes, transport of the calcium across the cell mediated by CaBP and extrusion across the basolateral membranes of the intestinal epithelial cells by basolateral membrane CaATPase (33). In the intestine, 1,25-D₃ enhances calcium transport, in part, by stimulating vitamin D-dependent CaBPs mRNA expression and protein synthesis (13, 16, 18, 24-26), and enhancing epithelial cell CaATPase mRNA expression, protein synthesis and activity (11, 17, 19-22, 32, 35).

Our data indicate that administration of 1,25-D₃ to vitamin D deficient rats upregulate intestinal PMCA1 and CaBP-9K mRNA. The upregulation of PMCA1 mRNA by 1,25-D₃ peaked at 4 and 8 hours after injection and remained high at 12 hours. Also, 1,25-D₃ induced more prolonged induction of PMCA1 mRNA than did 1,25,28-D₂. Compared to that of PMCA1 mRNA expression, the response of CaBP-9K mRNA to 1,25-D₃ peaked at 8 and 12 hours after injection, which is in agreement

with earlier reports (1, 4, 6, 8, 35).

A report from Wang et al. (34) demonstrated that 12.5 ng of 1,25,28-D₂ did not have any effects on intestinal calcium absorption, intestinal CaBP-9K mRNA or CaBP-9K protein. Treatment with 250, 500 or 800 ng 1,25,28-D₂ resulted in significant induction of CaBP mRNA but still failed to increase intestinal calcium absorption. To the best of our knowledge, no reports on the induction of CaATPase mRNA by 1,25,28-D₂ have been documented. Our results from a single injection of 1,25,28-D₂ into vitamin D-deficient rats indicated that no induction of intestinal CaATPase and CaBP-9K mRNA occurred when 1 ug of 1,25,28-D₂ was used, but induction of both CaATPase mRNA and CaBP-9K was obvious after injection of 20 ug of 1,25,28-D₂ and this was accompanied by higher plasma calcium levels. The rapid transcriptional responses are probably due to the binding of hormone-receptor complexes to, and the cellular responses from the responsive genes of vitamin D. The low affinity [0.8% of that of 1,25-D₃] of 1,25,28-D₂ to vitamin D receptors probably contributes, at least in part, to the experimental results in which large doses of the analog induced multiple cellular responses to elevate blood calcium concentrations. The rapid transcriptional response of both CaATPase and

CaBP-9K indicate that 1,25-D₃ is a primary regulator of calcium transport system in the intestine (9).

Acknowledgment

The authors wish to thank Dr. Richard L. Engen, Dr. Franklin A. Ahrens, Dr. Donald C. Beitz for all their support of the project. The authors also thank Derrel A. Hoy, Judy M. Pochop, Cynthia A. Hauber, K. C. Hummel, Mary Kassinos and Gary Fry for their technical support.

References

- 1. Armbrecht, H. J., Boltz, M. A. & Wongsurawat, N. 1994. Expression of plasma membrane calcium pump mRNA in rat intestine: effect of age and 1,25-dihydroxyvitamin D. *Biochim. Biophys. Acta* 1195:110-114.**
- 2. Aviv, H. & Leder, P. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA* 69:1408-1412.**
- 3. Bronner, F. 1992. Current concepts of calcium absorption: an overview. *J. Nutr.* 122:641-643.**
- 4. Cai, Q., Chandler, J. S., Wasserman, R. H., Kumar, R. & Penniston, J. T. 1993. Vitamin D and adaptation to dietary calcium and phosphate deficiencies increase intestinal plasma membrane calcium pump gene expression. *Proc. Natl. Acad. Sci. USA* 90:1345-1349.**

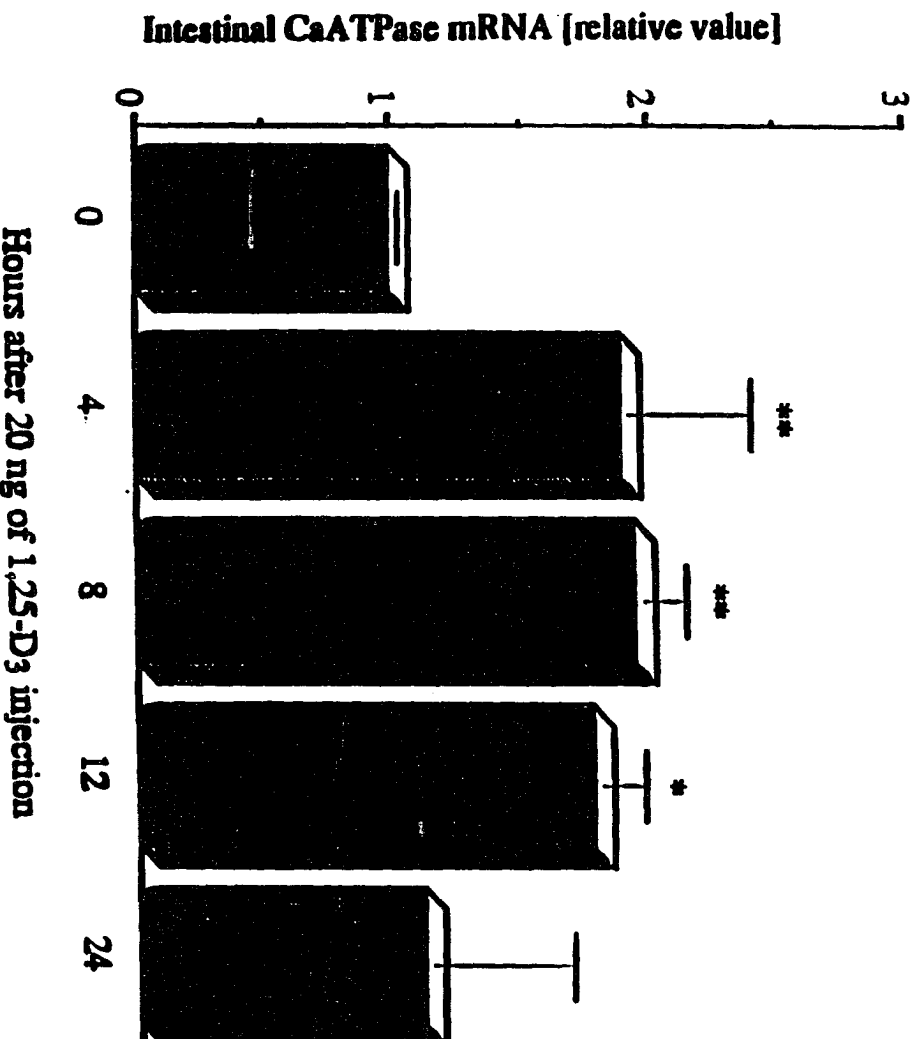
- 5. Chabanis, S., Hanrotel, C., Duchambon, P., Banide, H., Kubrusly, M., Aymard, P., Lacour, B. & Drueke, T. 1994. Duodenal calcium binding protein and active transport in rats: are they functionally related? Nephrol. Dial. Transplant. 9:1402-1407.**
- 6. Christakos, S., Gabrielides, C. & Rhoten, W. B. 1989. Vitamin D-dependent calcium binding proteins: chemistry, distribution, functional considerations, and molecular biology. Endocrine Rev. 10:3-16.**
- 7. Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J. & Kirschner, M. W. 1980. Number and evolutionary conservation of a α and β tubulin and cytoplasmic β and γ actin genes using specific cloned cDNA probes. Cell. 20:95-105.**
- 8. Darwish, H. M. & DeLuca, H. F. 1992. Identification of a 1,25-dihydroxyvitamin D₃ response element in the 5'-flanking region of rat calbindin D-9K gene. Proc. Natl. Acad. Sci. U. S. A. 89:603-607.**
- 9. DeLuca, H. F. 1988. The vitamin story: a collaborative effort of basic science and clinical medicine. FASEB J. 2:224-236.**
- 10. Favus, M. J., Tembe, V., Ambrosic, K. A. & Nellans, H. N. Effects of 1,25-dihydroxyvitamin D₃ on enterocyte basolateral membrane calcium transport in rats. 1989. Am. J. Physiol. G613-G617.**
- 11. Ghijsen, W. E. & Van Os C. H. 1982. 1,25-dihydroxyvitamin D₃ regulates ATP-dependent calcium transport in basolateral plasma membranes of rat enterocytes. Biochim. Biophys. Acta. 689:170-172.**
- 12. Ghijsen, W. E., Dejong, M. D. & van Os, C. H. 1982. ATP-dependent calcium transport and its correlation with Ca²⁺-ATPase activity in basolateral plasma membranes of rat duodenum. Biochim. Biophys. Acta. 689:327-336.**
- 13. Gross, M. & Kumar, R. 1990. Physiology and biochemistry of vitamin D-dependent calcium binding proteins. Am. J. Physiol. 259:F195-F209.**

- 14. Ham, J. and Parker, M. G. 1989. Regulation of gene expression by nuclear hormone receptors. *Curr. Opin. Cell Biol.* 1:503-511.**
- 15. Haussler, M. R. 1986. Vitamin D receptors: nature and function. *Annu. Rev. Nutr.* 6:527-562.**
- 16. Heimann, C. W. & Hunziker, H. 1990. Intracellular calcium binding molecules. In: Bronner, F. (ed) *Intracellular calcium regulation.* Alan. R. Liss Inc., New York, p 211.**
- 17. Kumar, R. 1986. The metabolism and mechanism of action of 1,25-dihydroxyvitamin D₃. *Kidney Int.* 30:793-803.**
- 18. Kumar, R. 1991. Vitamin and calcium transport. *Kid. Int.* 40:1177-1189.**
- 19. Lane, S. M. & Lawson, D. E. M. 1978. Differentiation of the changes in alkaline phosphatase from calcium ion-activated adenosine triphosphatase activities associated with increased calcium absorption in chick intestine. *Biochem. J.* 174:1067-1070.**
- 20. Martin, D. L., Melancon, M. J. & DeLuca, H. F. 1969. Vitamin D stimulated, calcium-dependent adenosine triphosphatase from brush borders of rat small intestine. *Biochem. Biophys. Res, Commun.* 35:819-823.**
- 21. Marx, S. J., Liberman, U. A. & Eil, C. 1983. Calciferols: actions and deficiencies of action. *Vitam. Horm.* 40:235-308.**
- 22. Melancon, M. J. & DeLuca, H. F. 1970. vitamin D stimulation of calcium-dependent adenosine triphosphatase in chick intestinal brush borders. *Biochemistry.* 9:1658-1664.**
- 23. Minghetti, P. P. & Norman, A. W. 1988. 1,25-dihydroxyvitamin D₃ receptors: gene regulation and genetic circuitry. *FASEB J.* 2:3043-3053.**

24. Norman, A. W., Roth, J. & Orci, L. 1982. The vitamin D endocrine system: steroid metabolism, hormone receptors and biological response (calcium binding proteins). *Endoc. Rev.* 3:331-366.
25. Pavlovitch, J. H., Didierjean, L., Rizk, M., Balsan, S. & Saurat, J. H. 1983. Skin calcium-binding protein: distribution in other tissues. *Am. J. Physiol.* 244:C50-C57.
26. Schelling, C. P., Didierjean, L., Rizk, M., Panlovitch, J. H., Takagi, T. & Heimann, C. W. 1987. Calcium-binding proteins in rat skin. *FEBS Lett.* 214:21-27.
27. Takito, J., Shinki, T., Tanaka, H. & Suda, T. 1992. Mechanism of regulation of calcium pumping activity in chick intestine. *Am. J. Physiol.* 262:G797-G805.
28. Timmermans, J. A., Kaune, R., Bindels, R. J. & van Os, C. H. 1991. Quantification of $\text{Ca}^{(2+)}$ -ATPases in porcine duodenum. Effect of 1,25-dihydroxyvitamin D_3 deficiency. *Biochim. Biophys. Acta* 1065:177-184.
29. Wasserman, R. H. 1981. Intestinal absorption of calcium and phosphorus. *Fed. Proc.* 40:68-72.
30. Wasserman, R. H., Fullmer, C. S. & Shimura, F. 1984. Calcium absorption and the molecular effects of vitamin D_3 , in *Vitamin D: basic and clinical aspects*, edited by Kumar, R., Boston, Martinus Nijhoff. p 233-302.
31. Wasserman, R. H., Chandlwer, J. S., Meyer, S. A., Smith, C. A., Brindak, M. E., Fullmer, C. S., Penniston, J. T. & Kumar, R. 1992. Intestinal calcium transport and calcium extrusion processes at basolateral membrane. *J. Nutr.* 122:662-671.
32. Wasserman, R. H., Smith, C. AS., Brindak, M. E., Talamoni, N. D., Fullmer, C. S., Penniston, J. T. & Kumar, R. 1992. Vitamin and mineral deficiencies increase the plasma membrane calcium pump of chick intestine. *Gastroenterology.* 102:886-894.

- 33. Wasserman, R. H. & Fullmer, C. S. 1995. Vitamin D and intestinal calcium transport: facts, speculations and hypotheses. J. Nutr. 125:1971S-1979S.**
- 34. Wang, Y. Z., Li, H., Bruns, M. E., Uskokovic, M., Truitt, G. A., Horst, R., Reinhardt, T., and Christakos, S. 1993. Effect of 1,25,28-trihydroxyvitamin D₂ and 1,24,25-trihydroxyvitamin D₃ on intestinal calbindin-D_{9K} mRNA and protein: Is there a correlation with intestinal calcium transport? J. Bone Miner. Res. 8:1483-1490.**
- 35. Zelinski, J. M., Sykes, D. E. & Weiser, M. M. 1991. The effect of vitamin D on rat intestinal plasma membrane Ca-pump mRNA. 179:749-755.**

Appendix with mean data tables

Figure 1. Intestinal CaATPase mRNA expression after 1,25-D₃ injection

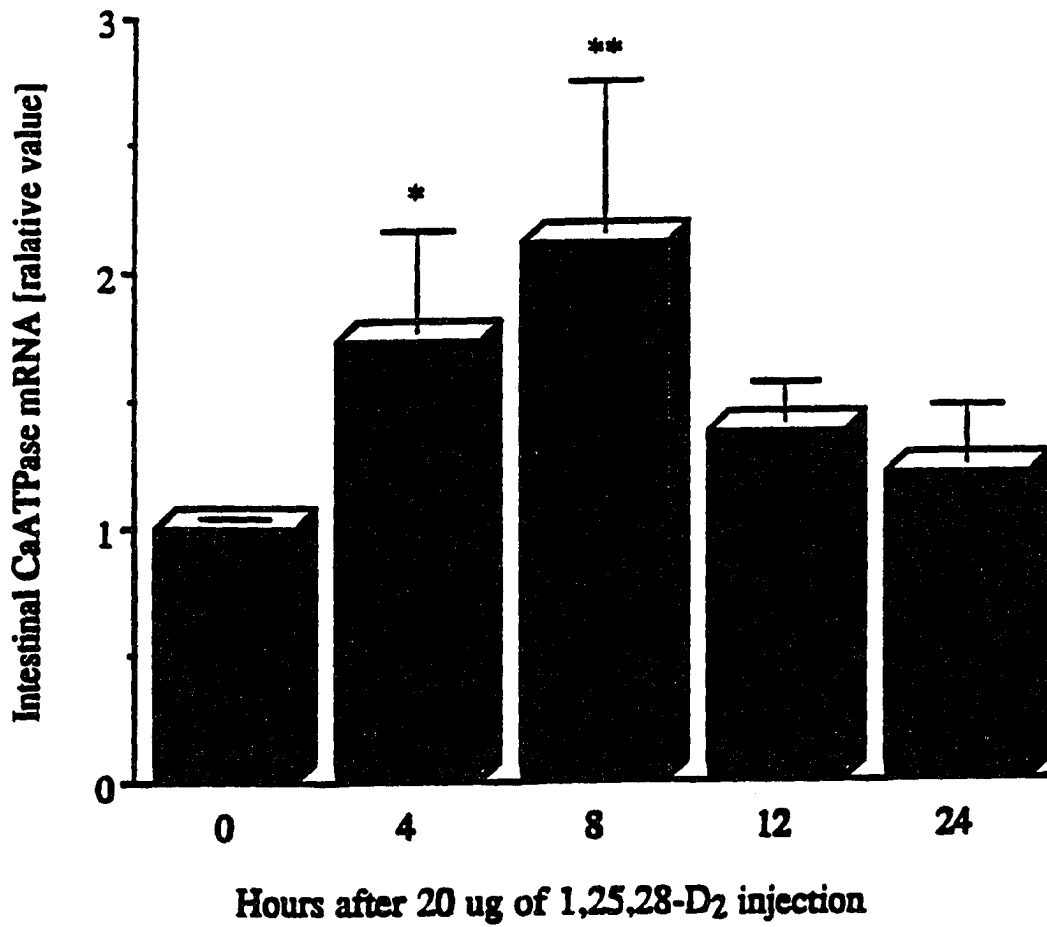


Figure 2. Intestinal CaATPase mRNA expression after 1,25,28-D₂ injection

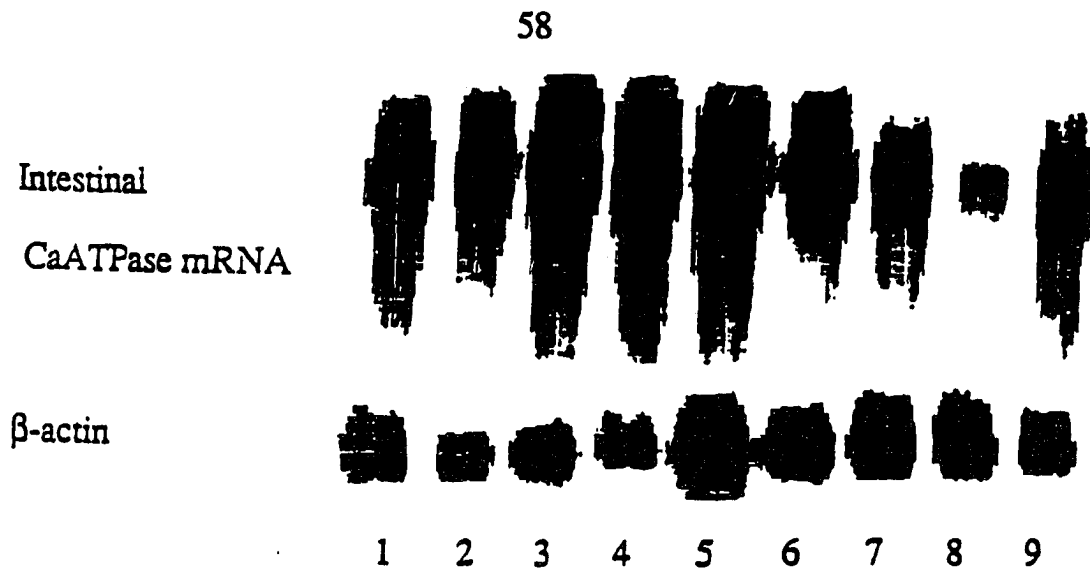


Figure 3. Northern blot of intestinal CaATPase mRNA

- 1. Control**
- 2. 4 hours after 20 ng of 1,25-D₃ injection**
- 3. 4 hours after 20 ug of 1,25,28-D₂ injection**
- 4. 8 hours after 20 ng of 1,25-D₃ injection**
- 5. 8 hours after 20 ug of 1,25,28-D₂ injection**
- 6. 12 hours after 20 ng of 1,25-D₃ injection**
- 7. 12 hours after 20 ug of 1,25,28-D₂ injection**
- 8. 24 hours after 20 ng of 1,25-D₃ injection**
- 9. 24 hours after 20 ug of 1,25,28-D₂ injection**

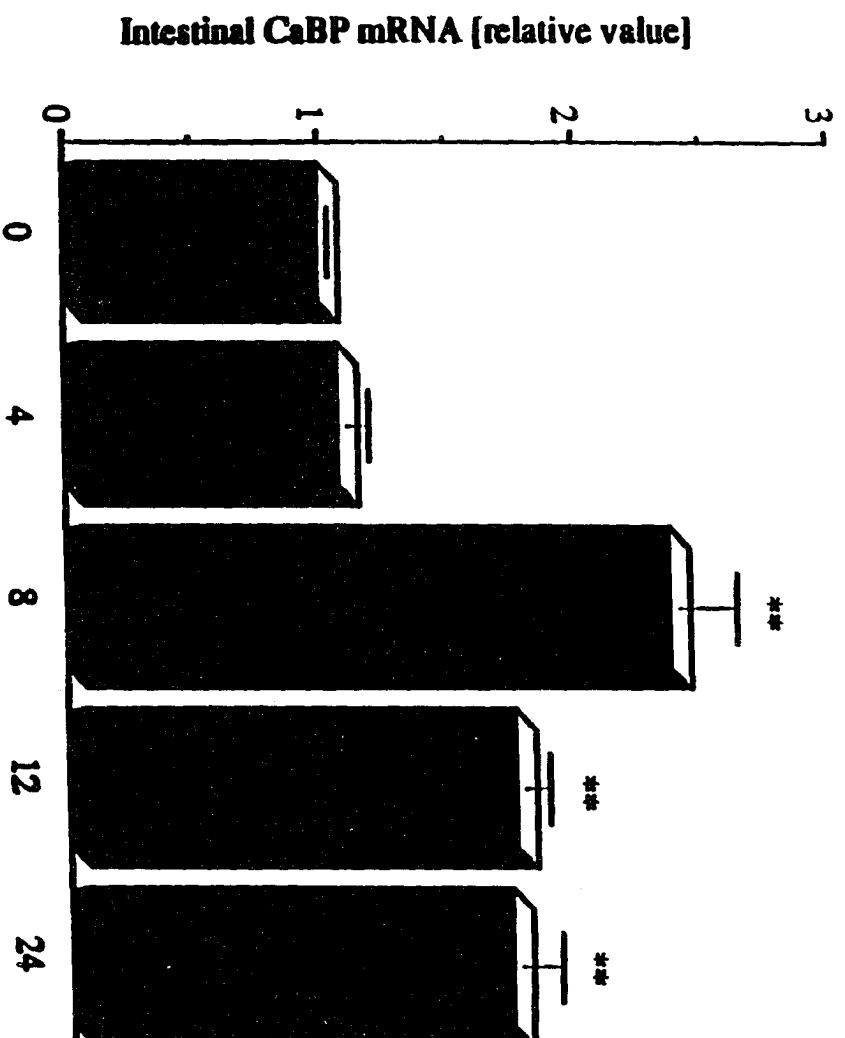


Figure 4. Intestinal CaBP mRNA expression after 1,25-D₃ injection

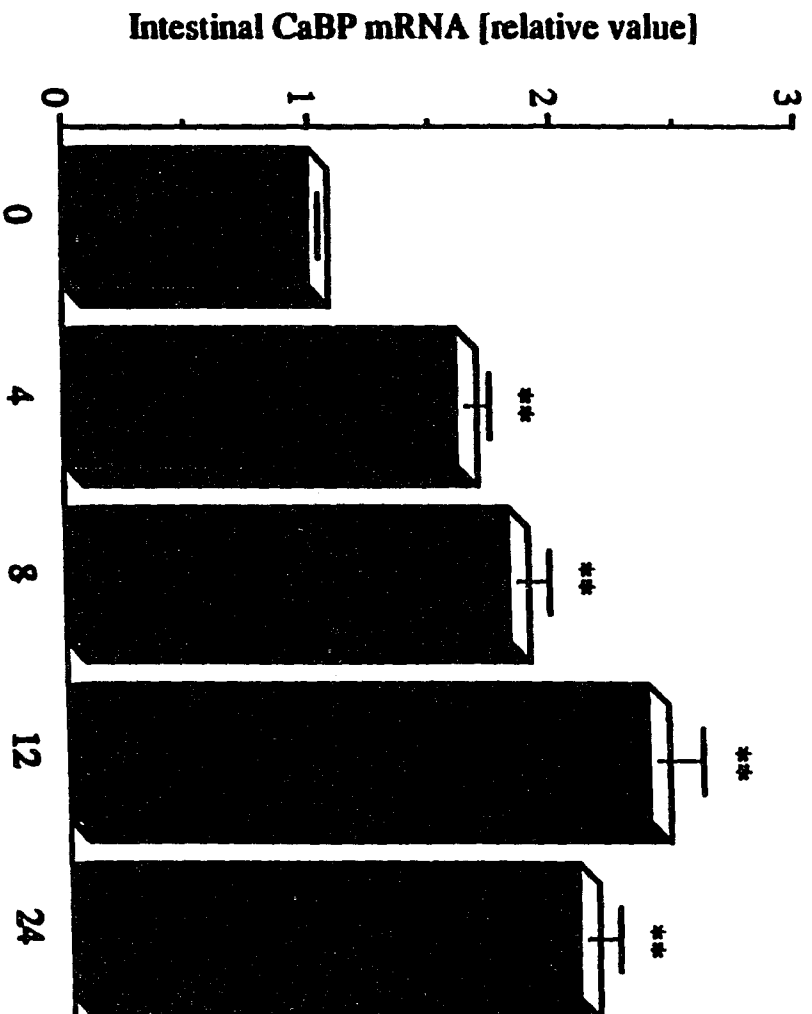


Figure 5. Intestinal CaBP mRNA expression after 1,25,28-D₂ injection

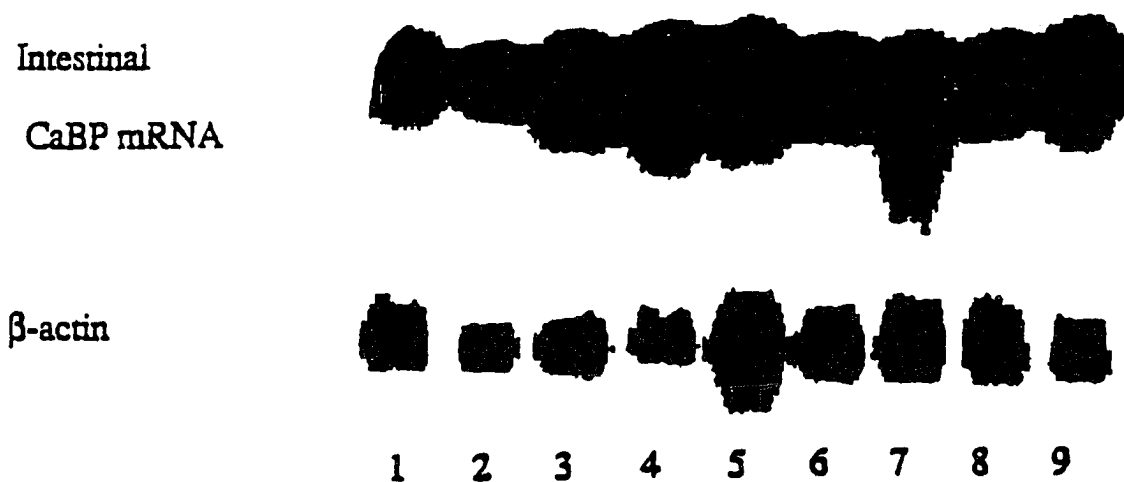


Figure 6. Northern blot of intestinal CaBP mRNA

- 1. Control**
- 2. 4 hours after 20 ng of 1,25-D₃ injection**
- 3. 4 hours after 20 ug of 1,25,28-D₂ injection**
- 4. 8 hours after 20 ng of 1,25-D₃ injection**
- 5. 8 hours after 20 ug of 1,25,28-D₂ injection**
- 6. 12 hours after 20 ng of 1,25-D₃ injection**
- 7. 12 hours after 20 ug of 1,25,28-D₂ injection**
- 8. 24 hours after 20 ng of 1,25-D₃ injection**
- 9. 24 hours after 20 ug of 1,25,28-D₂ injection**

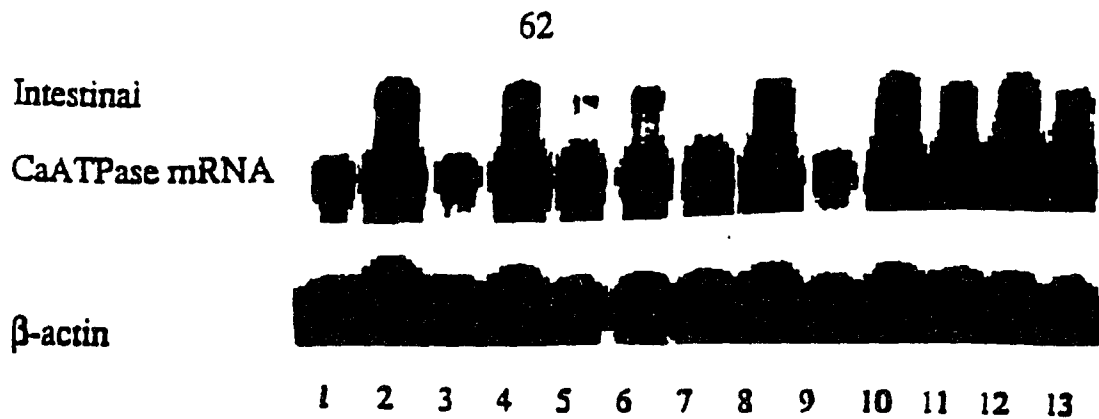


Figure 7. Northern blot of intestinal CaATPase mRNA

1. Control
2. 4 hours after 20 ng of 1,25-D₃ injection
3. 4 hours after 1 ug of 1,25,28-D₂ injection
4. 8 hours after 20 ng of 1,25-D₃ injection
5. 8 hours after 1 ug of 1,25,28-D₂ injection
6. 12 hours after 20 ng of 1,25-D₃ injection
7. 12 hours after 1 ug of 1,25,28-D₂ injection
8. 16 hours after 20 ng of 1,25-D₃ injection
9. 16 hours after 1 ug of 1,25,28-D₂ injection
10. 24 hours after 20 ng of 1,25-D₃ injection
11. 24 hours after 1 ug of 1,25,28-D₂ injection
12. 36 hours after 20 ng of 1,25-D₃ injection
13. 36 hours after 1 ug of 1,25,28-D₂ injection

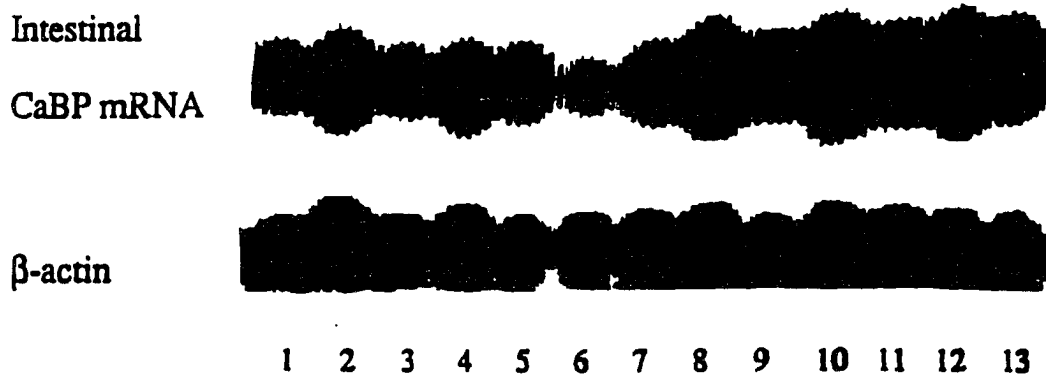


Figure 8. Northern blot of intestinal CaBP mRNA

1. Control
2. 4 hours after 20 ng of 1,25-D₃ injection
3. 4 hours after 1 ug of 1,25,28-D₂ injection
4. 8 hours after 20 ng of 1,25-D₃ injection
5. 8 hours after 1 ug of 1,25,28-D₂ injection
6. 12 hours after 20 ng of 1,25-D₃ injection
7. 12 hours after 1 ug of 1,25,28-D₂ injection
8. 16 hours after 20 ng of 1,25-D₃ injection
9. 16 hours after 1 ug of 1,25,28-D₂ injection
10. 24 hours after 20 ng of 1,25-D₃ injection
11. 24 hours after 1 ug of 1,25,28-D₂ injection
12. 36 hours after 20 ng of 1,25-D₃ injection
13. 36 hours after 1 ug of 1,25,28-D₂ injection

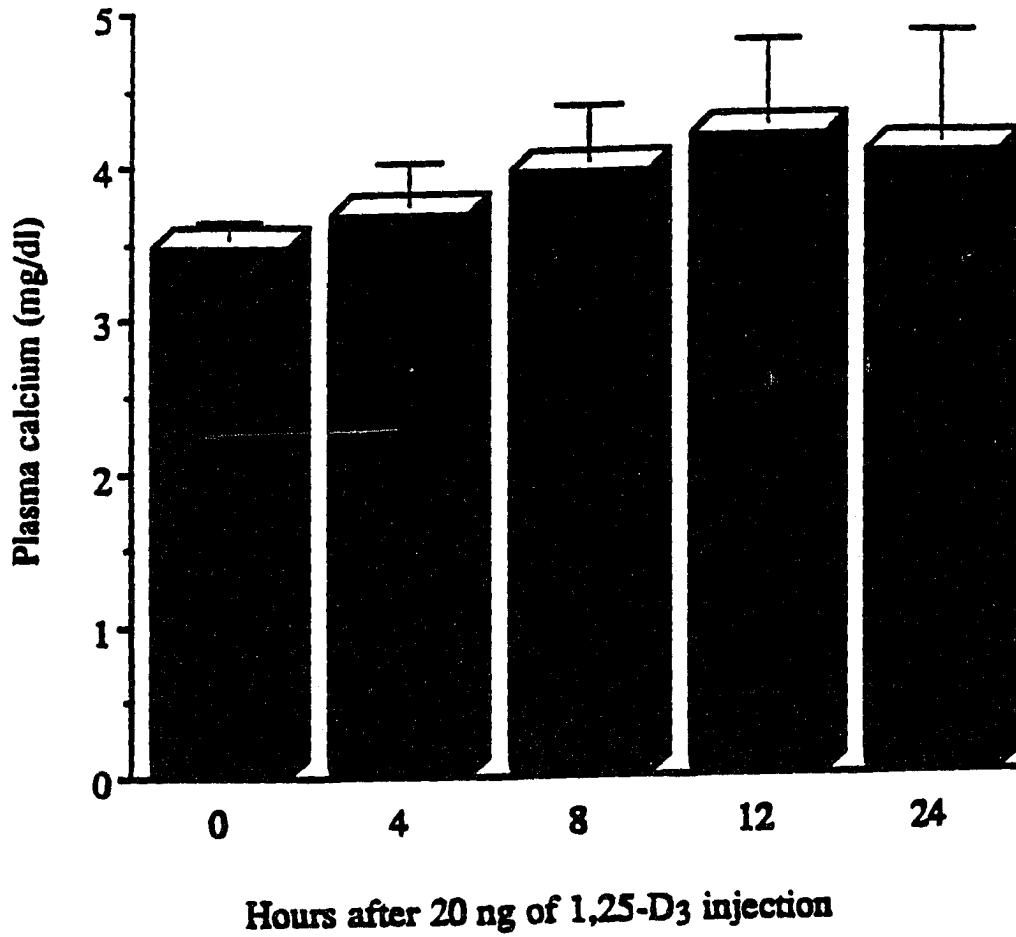


Figure 9. Plasma calcium concentration after 1,25-D₃ injection

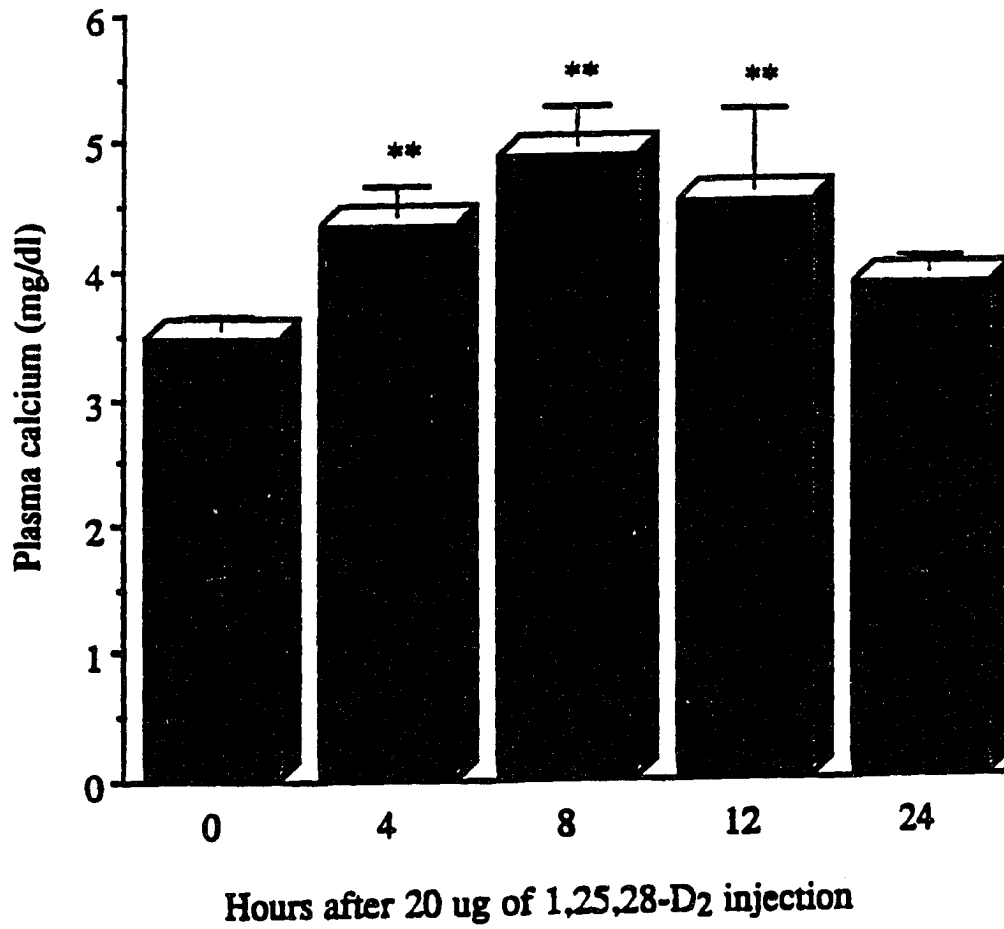


Figure 10. Plasma calcium concentration after 1,25,28-D₂ injection

Table 1. Intestinal CaATPase mRNA [relative value]

Hours after injection	1, 25-D3 treatment		1,25,28-D2 treatment	
	Mean	SD	Mean	SD
0	1.00	0	1.00	0
4	1.89**	0.48	1.73*	0.39
8	1.94**	0.17	2.11**	0.59
12	1.77*	0.16	1.36	0.16
24	1.12	0.54	1.20	0.22

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

Each group contains 4 rat samples.

Table 2. Intestinal CaBP mRNA [relative value]

Hours after injection	1, 25-D ₃ treatment		1,25,28-D ₂ treatment	
	Mean	SD	Mean	SD
0	1.00	0	1.00	0
4	1.07	0.08	1.61**	0.09
8	2.36**	0.22	1.81**	0.12
12	1.74**	0.10	2.37**	0.18
24	1.72**	0.16	2.07**	0.13

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

Each group contains 4 rat samples.

Table 3. Plasma calcium concentration [mg/dl]

Hours after injection	1, 25-D ₃ treatment		1,25,28-D ₂ treatment	
	Mean	SD	Mean	SD
0	3.48	0.10	3.48	0.10
4	3.69	0.27	4.34**	0.23
08	3.95	0.35	4.87**	0.30
12	4.18	0.53	4.51**	0.62
24	4.05	0.71	3.86	0.12

* p<0.05 compared to control group

** p<0.01 compared to control group

Each group contains 4 rat samples.

EFFECT OF DIETARY CALCIUM AND VITAMIN D RESTRICTION ON RAT INTESTINAL CALCIUM ATPase AND CALCIUM BINDING PROTEIN mRNA EXPRESSION

A paper to be submitted to the J. of Bone and Miner. Res.

Yingting Zhu, Jesse P. Goff, Timothy A. Reinhardt and Ronald L. Horst

Abstract

Vitamin D deficiency prevents the active transport of calcium across the intestine. Modulation of calcium pump and calcium binding protein gene expression by dietary vitamin D and calcium restriction is still not well understood. In this study, the effects of dietary calcium and/or vitamin D-deficiency on intestinal plasma membrane calcium ATPase [PMCA1] and calcium binding protein [CaBP-9K] mRNA expression in rats was examined. The results indicate both intestinal PMCA1 and CaBP-9K mRNA are downregulated in rats fed diets containing no vitamin D. However, dietary calcium restriction alone did not increase CaATPase or CaBP-9K mRNA. This study demonstrates that dietary calcium itself is probably not a major regulator in mRNA expression of calcium

transporting factors such as PMCA1 and CaBP-9K when physiological doses of vitamin D are supplied in diet.

Introduction

Dietary calcium absorption in the intestine can be transcellular or paracellular (6). The transcellular movement is dominant in the small intestine (27). In transcellular movement, the entry of calcium into cells can occur by simple diffusion across the plasma membrane and is dependent on the electrochemical gradient of calcium through calcium channels or exchangers (1, 38). After entry into the epithelial cell, calcium binding protein transports calcium to the basolateral membrane where CaATPase pumps calcium out of the cell against its concentration gradient into the extracellular fluids (39).

It is well known that 1,25-dihydroxyvitamin D₃ [125-D₃] is a primary regulator of intestinal calcium absorption (24, 27, 36). The vitamin stimulates calcium transport by classic hormone-receptor-mediated processes (10, 24). 1,25-D₃ markedly enhances CaATPase mRNA concentration and activity and elevates CaBPs and their mRNA (14, 15, 17-22, 26, 30, 41).

Whether calcium restriction induces upregulation of CaATPase and CaBP is controversial. Favus et al. (12) reported that the intestinal calcium transport in rats adapted to a calcium-deficient diet was decreased (12). Brehier (5) indicated that calcium induced a 6- to 10-fold increase of CaBP-9K mRNA, which could be blocked by actinomycin D in duodenal organ culture. deBoland and Norman (9) demonstrated that stimulation of duodenal calcium transport in chicks by 1,25-D₃ was dependent on the presence of calcium in the media, since transport was abolished by prior addition of EGTA and was restored upon addition of calcium. Meyer et al. (23) reported that reduced dietary calcium increased intestinal CaBP mRNA, but at the same time diminished vitamin D receptor [VDR] mRNA despite increased circulating 1,25-D₃. Theofan et al. (37) found that there was no difference in steady state intestinal CaBP mRNA levels between rats fed low or normal calcium diet. Hall and Norman (16) indicated that, in vitamin D deficient rats, dietary calcium manipulation did not affect intestinal CaBP or its mRNA in chicks; however, a single injection of 1,25-D₃ induced both duodenal CaBP and CaBP mRNA. Some earlier reports suggested that the adaptation to a calcium-deficient diet was associated with increased CaBPs and CaBP mRNA (3, 4, 13, 25, 29, 31, 34). Moreover,

Wasserman et al. (39, 40) found that mineral deficiencies increased the plasma membrane CaATPase of chick intestine. Cai et al. (7) claimed that adaptation to dietary calcium deficiencies increased intestinal CaATPase gene expression. These discrepancies suggest that it is important to evaluate whether the adaptation to calcium deficiencies can induce gene expression of intestinal calcium transporting factors such as CaATPase and CaBP.

Materials and Methods

Animals: In trial 1, three-week old Holtzman Sprague-Dawley weaning rats were placed on four different synthetic diets, i.e., +Ca, +D diet [1% calcium, 5 IU vitamin D/g] (Tekland, Madison, WI); 2. -Ca, -D diet [0.002 % calcium, 0 IU vitamin D/g]; 3. -D diet [0.5% calcium, 0 IU vitamin D/g]; 4. -Ca diet [0.002% calcium, 5 IU vitamin D/g] for three weeks to induce vitamin D- and/or calcium-deficient state. In trial 2, the rats were fed a normal diet or a -Ca diet for 1 week, 2 weeks or 3 weeks. Rats were killed by inhalation of CO₂-O₂ [50:50] followed by exsanguination. Blood samples were collected into heparinized tubes. The proximal 15 cm of duodenal mucosa was collected from each rat and

frozen for later mRNA analysis.

Northern blotting: Intestinal CaATPase, CaBP and VDR mRNA levels were determined by Northern blotting. Total RNA was isolated by TRIzol (GIBCO, BRL, Gaithersburg, MD) and chloroform, precipitated and washed by isopropanol and ethanol. PolyA-containing RNA was selected by oligo dT cellulose affinity chromatography (2). RNA was fractionated on 1.2% formaldehyde agarose gel and transferred to nylon membranes (MSI Micro Separations Inc., Westboro, MA). The RNA was cross-linked by Stratalinker 1800 (Stratagene, La Jolla, CA). A 3.4 kb cDNA from rat brain plasma membrane CaATPase [PMCA1], kindly supplied by Dr. Gary Shull, University of Cincinnati, OH, was used to hybridize the membranes for analysis of CaATPase mRNA. The cDNA for CaBP-9K mRNA assay was a gift of Dr. M. Elizabeth Bruns, University of Virginia Medical School, Charlottesville, VA.

Membranes were prehybridized at 42^o C for 4-6 hours in a prehybridization buffer containing 50% formamide, 5X Denhardt's reagent, 0.1% sodium dodecyl sulfate [SDS] and 100 ug/ml denatured fish sperm DNA. Fresh prehybridization buffer and 250 ug tRNA was used for the overnight membrane hybridization with 5 x 10⁶ cpm ³²P-labeled

cDNA probe/ml prehybridization buffer. The membranes were washed twice in 2X SSPE/0.1% SDS buffer [SSPE is NaCl, NaH₂PO₄, EDTA mixture], twice in 0.1X SSPE/0.1% SDS, each for 10 minutes.

Quantitative analysis of mRNA was done by Electronic Autoradiography (PACKARD, Meriden, CT). A 2.1 kb chick β -actin cDNA probe was obtained by digestion of pBR322 with Hind III (8) and used as a control for estimation of mRNA integrity and RNA sample loading difference among various preparations.

Plasma 1,25-D₃ determination: The method of Reinhardt et al (32) was modified to determine 1,25-D₃ concentration in plasma. The plasma 1,25-D₃ was chromatographed on C18/OH low hydrocarbon columns (Varian, Harbor City, CA), and the concentration was determined by radio-receptor assay. Recovery of [³H]1,25-D₃ through the purification steps for each sample allowed correction for extraction efficiency.

Plasma calcium concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer Corp., Norwalk, CT).

Analysis of variance was used to determine if differences among CaATPase, CaBP, VDR mRNA and plasma constituents as a result of different dietary treatment. When ANOVA suggested differences existed,

Dunnnett multiple comparisons test was used to test the significance between each dietary treatment.

Results

In trial 1, intestinal CaATPase and CaBP mRNA decreased significantly in the duodenum of rats fed -D diets, regardless of dietary calcium (Figure 1, 2, 3, 4, Table 1 and 2). Intestinal CaATPase and CaBP-9K mRNA levels were unaffected by dietary calcium treatment in vitamin D-replete rats (Figure 1, 2, 3, 4, Table 1 and 2). In trial 2, intestinal calcium ATPase mRNA was unaffected in rats after 1, 2 and 3 weeks of dietary calcium restriction (Figure 5). CaBP mRNA did not increase after 1, 2, 3 weeks of calcium restriction (Figure 6). No increase of either CaATPase or CaBP mRNA was observed, despite the significantly higher level of plasma 1,25-D₃ in rats fed -Ca diet . Plasma 1,25-D₃ concentrations in all rats fed -D diet with 0% calcium or 1% calcium were low (Figure 7 and Table 3). Plasma calcium levels in vitamin D-deficient rats were lower than those in vitamin D-replete rats, regardless of dietary calcium. Plasma calcium levels in rats fed -Ca diet were significantly lower than those in rats fed 1% calcium diet, but higher than

those in rats fed vitamin D-deficient diets (Figure 8 and Table 4).

Discussion

Our results clearly demonstrate that vitamin D deficiency with or without calcium deficiency induced similar downregulation of both intestinal CaATPase and CaBP mRNA, indicating that vitamin D, not calcium, is a major regulator of active calcium transporting factors (11, 24, 27, 34, 36). Dietary calcium itself did not seem to play a major role in gene transcription of CaATPase and CaBP, but the published papers on this topic are contradictory (3-5, 7, 9, 12, 13, 16, 23, 25, 29, 31, 35, 37, 40). Recently, Meyer et al (7) and Cai et al (23) reported that restriction of dietary calcium could induce upregulation of CaBP and CaATPase mRNA. However, our experiments failed to demonstrate upregulation of CaATPase or CaBP mRNA, implying that restriction of calcium may not induce adaptive upregulation of gene transcription of calcium transporting factors. This is in agreement with recent reports in which negative results of calcium deficiencies on the gene expression of CaATPase and CaBP have been reported (5, 12, 16, 37). The significantly higher plasma 1,25-D₃ in rats fed -Ca diet failed to upregulate vitamin D dependent CaATPase and

CaBP-9K mRNA. Whether the hypocalcemia counteracts the action of increased 1,25-D₃ is unknown (9, 33). Higher levels of plasma calcium in rats fed -Ca diet, compared to that in rats fed -D diet, also suggests vitamin D, not calcium, is a primary regulator of calcium homeostasis (24, 27, 36). The mechanism by which intestinal calcium transport is increased under conditions of low dietary calcium concentration remains questionable and needs further studies.

Acknowledgment

The authors wish to thank Dr. Richard L. Engen, Dr. Franklin A. Ahrens, Dr. Donald C. Beitz for all their support of the project. The authors also thank Derrel, A. Hoy, Judy M. Pochop, Cynthia, A. Hauber, K. C. Hummel, Mary Kassinos, and Gary Fry for their technical support.

References

1. Adams, D. J., Barakeh, J., Laskey, R., & Van Breemen, C. 1989. Ion Channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB J.* 3:2389-2400.
2. Aviv, H. & Leder, P. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA* 69:1408-1412.

- 3. Bar, A. & Wasserman, R. H. 1973. Control of calcium absorption and intestinal binding protein synthesis. *Biochem. Biophys. Res. Commun.* 54:191-196.**
- 4. Bar, A. & Hurwitz, S. 1979. The interaction between dietary calcium and gonadal hormones in their effect on plasma calcium, bone, 25-hydroxycholecalciferol-1-hydroxylase, and duodenal calcium-binding protein, measured by a radioimmunoassay in chicks. *Endocrinology.* 104:1455-1460.**
- 5. Brehier, A. & Thomasset, M. 1990. Stimulation of calbindin-D9K (CaBP-9K) gene expression by calcium and 1,25-dihydroxyvitamin D₃ in fetal duodenal organ culture. *Endocrinology.* 127:580-587.**
- 6. Bronner F. 1992. Current concepts of calcium absorption: An overview. *J. Nutr.* 122:641-643.**
- 7. Cai, Q., Chandler, J. S., Wasserman, R.H., Kumar, R., Penniston, J. T. 1993. Vitamin D and adaptation to dietary calcium and phosphate deficiencies increase the intestinal plasma membrane calcium pump gene expression. *Proc. Natl. Acad. Sci.* 90:1345-1349.**
- 8. Cleverland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J. & Kirschner, M. W. 1980. Number and evolutionary conservation of a α and β tubulin and cytoplasmic b and g actin genes using specific cloned cDNA probes. *Cell.* 20:95-105.**
- 9. deBoland, A. R. & Norman, A. W. 1990. Influx of extracellular calcium mediates 1,25-dihydroxyvitamin D₃-dependent transcalcitachia (the rapid stimulation of duodenal Ca²⁺ transport). *Endocrinology.* 127:2475-2480.**
- 10. DeLuca, H. F. & Schnoes, H. K. 1976. Metabolism and mechanism of action of vitamin D. *Annu. Rev. Biochem.* 45:631-666.**
- 11. DeLuca, H. F. 1988. The vitamin story: a collaborative effort of basic science and clinical medicine. *FASEB J.* 2:224-236.**

- 12. Favus, M. J., Tenbe, V., Ambrosic, K. A. & Nellans, H. N. 1989.** Effect of 1,25-dihydroxyvitamin D₃ on enterocyte basolateral membrane calcium transport in rats. *Am. J. Physiol.* 256:G613-G617.
- 13. Fox, J., Pickard, D. W., Care, A. D. & Murray, T. M. 1978.** Effect of low phosphorus diet on intestinal calcium absorption and the concentration of calcium-binding protein in intact and parathyroidectomized pigs. *Endocrinol.* 78:379-387.
- 14. Ghijssen, W. E. & van Os C. H. 1982.** 1,25-dihydroxyvitamin D₃ regulates ATP-dependent calcium transport in basolateral membranes of rat enterocytes. *Biochim. Biophys. Acta.* 689:170.
- 15. Gross, M. & Kumar, R. 1990.** Physiology and biochemistry of vitamin D-dependent calcium binding proteins. *Am. J. Physiol.* 259:F195-F209.
- 16. Hall, A. K. & Norman, A. W. 1990.** Regulation of calbindin-D28K gene expression in the chick intestine: effects of serum calcium status and 1,25-dihydroxyvitamin D₃. *J. Bone Miner. Res.* 5: 331-336.
- 17. Kumar, R. 1986.** The metabolism and mechanism of action of 1,25-dihydroxyvitamin D₃. *Kidney Int.* 30:793-803.
- 18. Kumar, R. 1991.** Vitamin D and calcium transport. *Kidney Int.* 40:1177-1189.
- 19. Lane, S. M. 1978.** Differentiation of the changes in alkaline phosphatase from calcium ion-activated adenosine triphosphatase activities associated with increased calcium absorption in chick intestine. *Biochem. J.* 174:1067-1070.
- 20. Martin, D. L., Melancon, M. J. & DeLuca, H. F. 1969.** Vitamin D stimulated, calcium-dependent adenosine triphosphatase from brush borders of rat small intestine. *Biochem. Biophys. Res. Commun.* 35:819-823.

- 21. Marx, S. J., Liberman, U. A. & Eil, C. 1983. Calciferols: actions and deficiencies of action. *Vitam. Horm.* 40:235-308.**
- 22. Melancon, M. J. & DeLuca H. F. 1970. Vitamin D stimulation of calcium-dependent adenosine triphosphatase in chick intestinal brush borders. *Biochem.* 9:1658-1664.**
- 23. Meyer, J., Fullmer, C. S., Wasserman, R. H., Komm, B. S. & Haussler, M. R. 1992. Dietary restriction of calcium, phosphorus and vitamin D elicits differential regulation of the mRNAs for avian intestinal calbindin-D28K and the 1,25-dihydroxyvitamin D receptor. *J. Bone and Miner. Res.* 7:441-448.**
- 24. Minghetti, P. P. & Norman, A. W. 1988. 1,25-dihydroxyvitamin D₃ receptors: gene regulation and genetic circuitry. *FASEB J.* 2:3043-3053.**
- 25. Morrissey, R. L. & Wasserman, R. H. 1971. Calcium absorption and calcium-binding protein in chicks on different calcium and phosphorus intakes. *Am J. Physiol.* 220:1509-1515.**
- 26. Norman, A. W., Roth, J. & Orci, L. 1982. The vitamin D endocrine system: steroid metabolism, hormone receptors and biological response (calcium binding protein). *Endoc. Rev.* 3:331-366.**
- 27. Norman, A. W. 1984. Hormonal actions of vitamin D. *Curr. Top. Cell Regul.* 24:35-49.**
- 28. Pansu, D., Bellaton, C., Roche, C. & Bronner, F. 1983. duodenal and ileal calcium absorption in the retard effects of vitamin D. *Am. J. Physiol.* 244:G695-G700.**
- 29. Pansu, D., Ballaton, C. & Bronner F. 1981. Effect of Ca intake on saturable and nonsaturable components of duodenal Ca transport. *Am. J. Physiol.* 240:G32-G37.**
- 30. Pavlovitch, J. H., Didierjean, L., Rizk, M., Balsan, S. & Saurat, J. H. 1983. Skin calcium protein: distribution in other tissues. *Am. J. Physiol.* 244:C50-C57.**

- 31. Rader, J. I., Baylink, D. J., Hughes, M., Safilian, E. F. & Haussler, M. R. 1979. Calcium and phosphorus deficiency in rats: effects on PTH and 1,25-dihydroxyvitamin D₃. *Am. J. Physiol.* 236:E118-E122.**
- 32. Reinhardt, T. A., Horst, R. L., Orf, J. W. & Hollis, B. W. 1984. A microassay for 1,25-dihydroxyvitamin D not requiring high performance liquid chromatography: application to clinical studies. *J. Clin. Endocrinol. Metab.* 59:91-98.**
- 33. Sandgren, M. E. & DeLuca, H. F. 1990. Serum calcium and vitamin D regulate 1,25-dihydroxyvitamin D₃ receptor concentration in rat kidney in vivo. *Proc. Natl. Acad. Sci. USA* 87:4312-4314.**
- 34. Strom, M., Krisinger, J., Darwish, H. & DeLuca, H. F. 1992. 1,25-dihydroxyvitamin D and not calcium is the major regulator of calbindin-D 9-kDA mRNA levels in vivo. *Proc. Soc. Exp. Biol. Med.* 199:369-371.**
- 35. Swaminathan, R., Sommerville, B. S. & Care, A. D. 1977. The effect of dietary calcium on the activity of 25-hydroxycholecalciferol-1-hydroxylase and Ca absorption in vitamin D-replete chicks. *Br. J. Nutr.* 38:47-54.**
- 36. Takito, J., Shinki, T., Tanaka, H. & Suda, T. 1992. Mechanism of regulation of calcium pumping activity in chick intestine. *Am. J. Physiol.* 262:G797-G805.**
- 37. Theofan, G., King, M. W., Hall, A. K. & Norman, A. W. 1987. Expression of calbindin-D28K mRNA as a function of altered serum calcium and phosphorus levels in vitamin D-replete chick intestine. *Mol. Cell Endocrinol.* 54:135-140.**
- 38. Wasserman, R. T. 1988. Cellular calcium and phosphate transport in health and disease, pp 3-13, Alan R. Liss, Inc.**
- 39. Wasserman, R. H., Chandler, J. S., Meyer, S. A., Smith, C. A., Brindak, M. E., Fullmer, C. S., Penniston, J. T. & Kumar,**

R. 1992. Intestinal calcium transport and calcium extrusion processes at basolateral membrane. *J. Nutr.* 122:662-671.

40. Wasserman, R. H., Smith, C. A., Brindak, M. E., Talamoni, N., Fullmer, C. S., Pennison, J. T. & Kumar, R. 1992. Vitamin D and mineral deficiencies increase the plasma membrane calcium of chick intestine. *Gastroenterology.* 102:886-894.

41. Zelinski, J. M., Sykes, D. E. & Weiser, M. M. 1991. The effect of vitamin D on rat intestinal plasma membrane Ca-pump mRNA. *Biochem. Biophys. Res. Commun.* 179:749-755.

Appendix with mean data tables

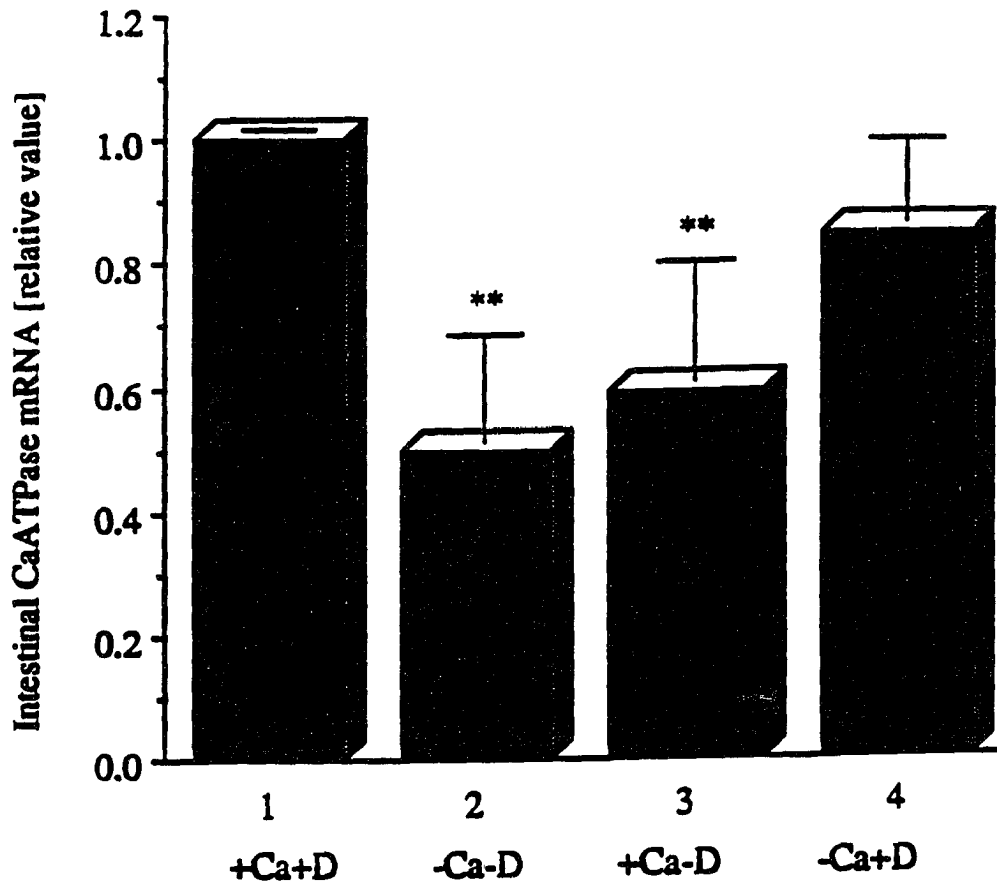


Figure 1. Intestinal CaATPase mRNA expression in rats fed different diets

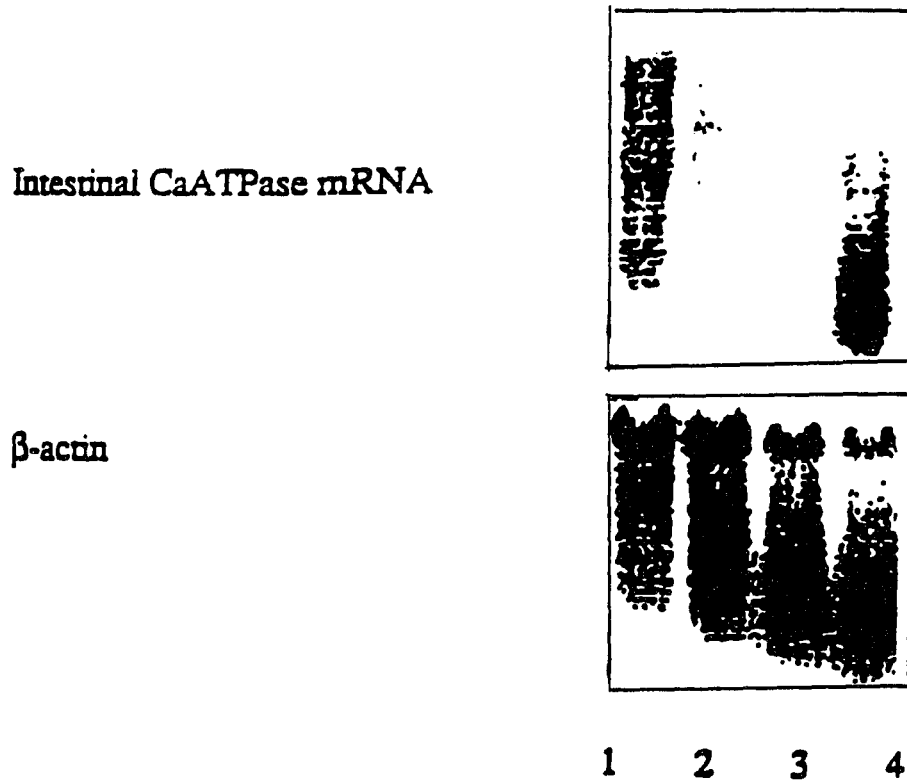


Figure 2. Northern blot of Intestinal CaATPase mRNA

- 1. +Ca+D diet treatment**
- 2. -Ca-D diet treatment**
- 3. +Ca-D diet treatment**
- 4. -Ca+D diet treatment**

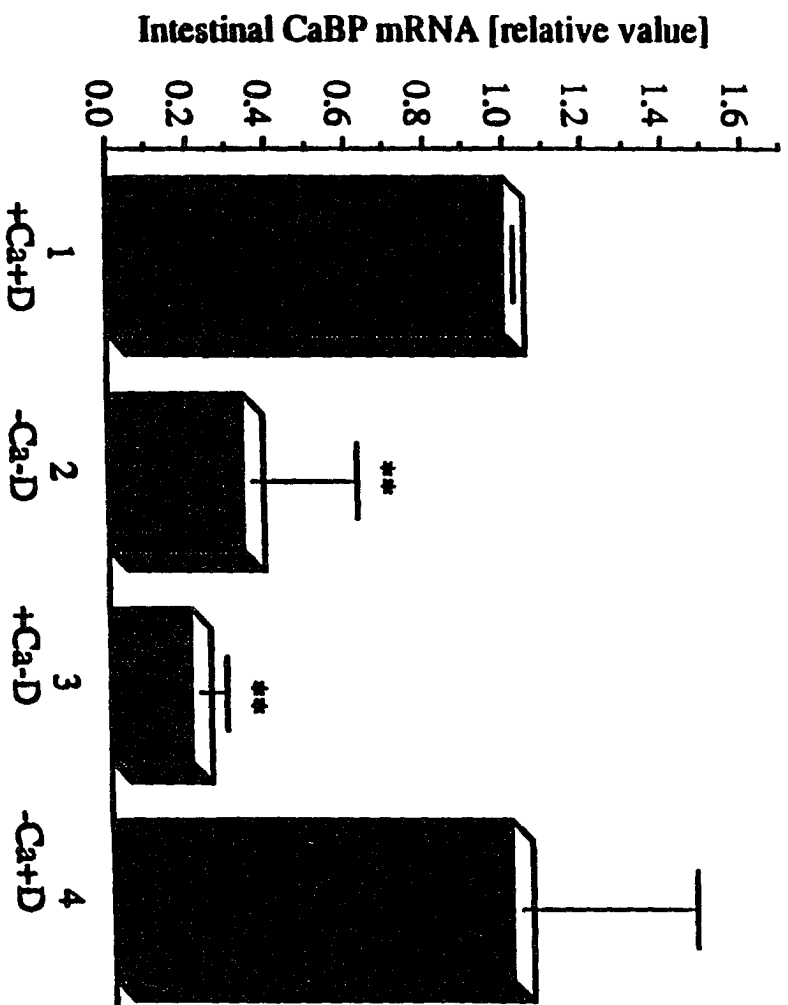


Figure 3. Intestinal CaBP mRNA expression in rats fed different diets

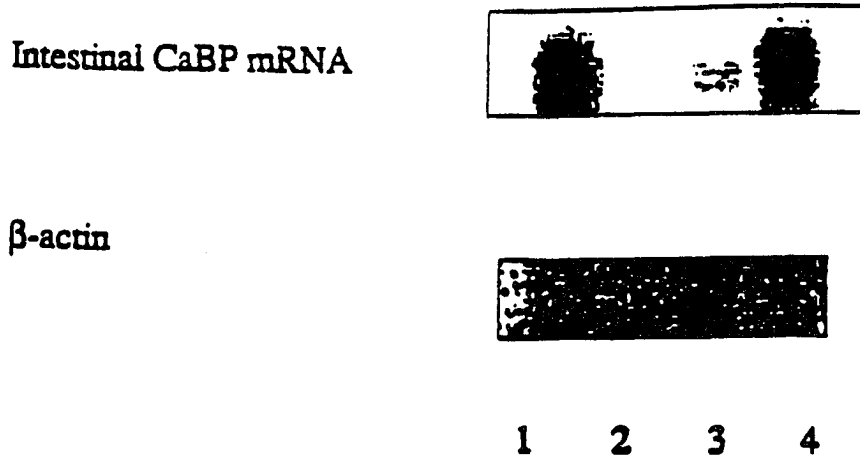


Figure 4. Northern blot of Intestinal CaBP mRNA

1. +Ca+D diet treatment
2. -Ca-D diet treatment
3. +Ca-D diet treatment
4. -Ca+D diet treatment

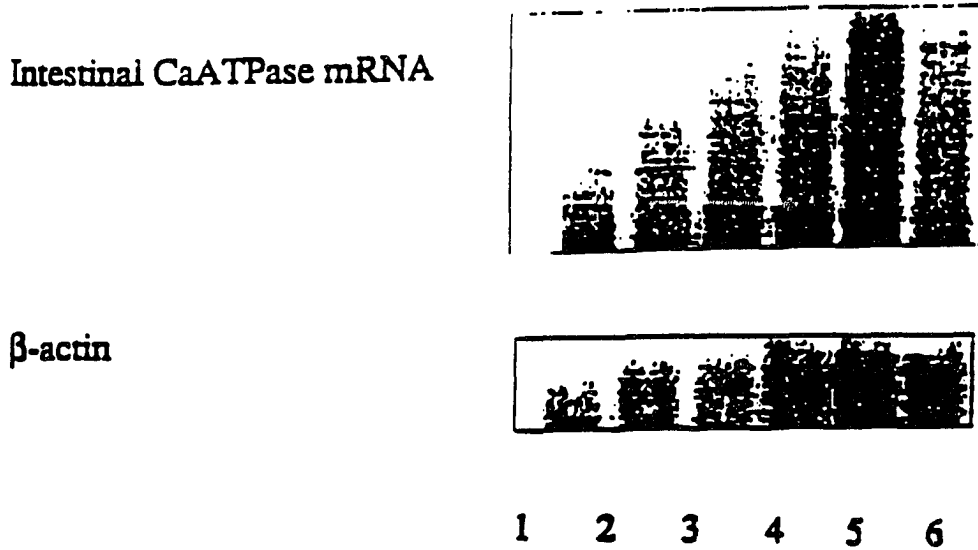


Figure 5. Northern blot of Intestinal CaATPase mRNA

1. +Ca+D diet treatment, 1 week
2. -Ca+D diet treatment, 1 week
3. +Ca+D diet treatment, 2 weeks
4. -Ca+D diet treatment, 2 weeks
5. +Ca+D diet treatment, 3 weeks
6. -Ca+D diet treatment, 3 weeks

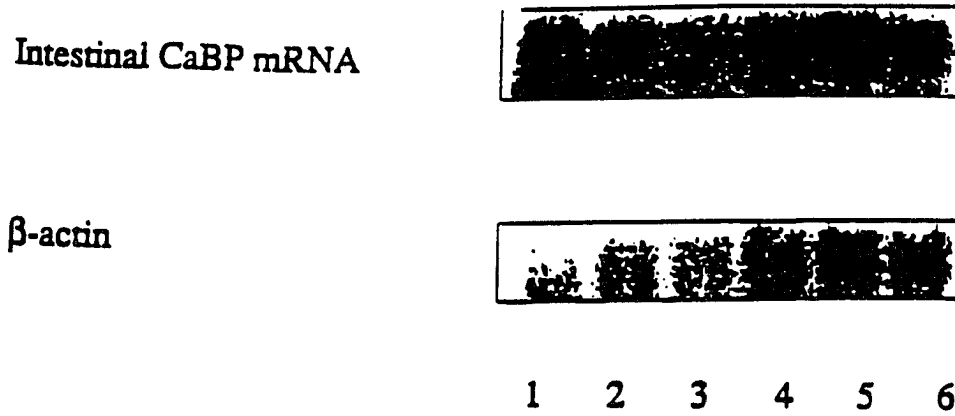


Figure 6. Northern blot of Intestinal CaBP mRNA

1. +Ca+D diet treatment, 1 week
2. -Ca+D diet treatment, 1 week
3. +Ca+D diet treatment, 2 weeks
4. -Ca+D diet treatment, 2 weeks
5. +Ca+D diet treatment, 3 weeks
6. -Ca+D diet treatment, 3 weeks

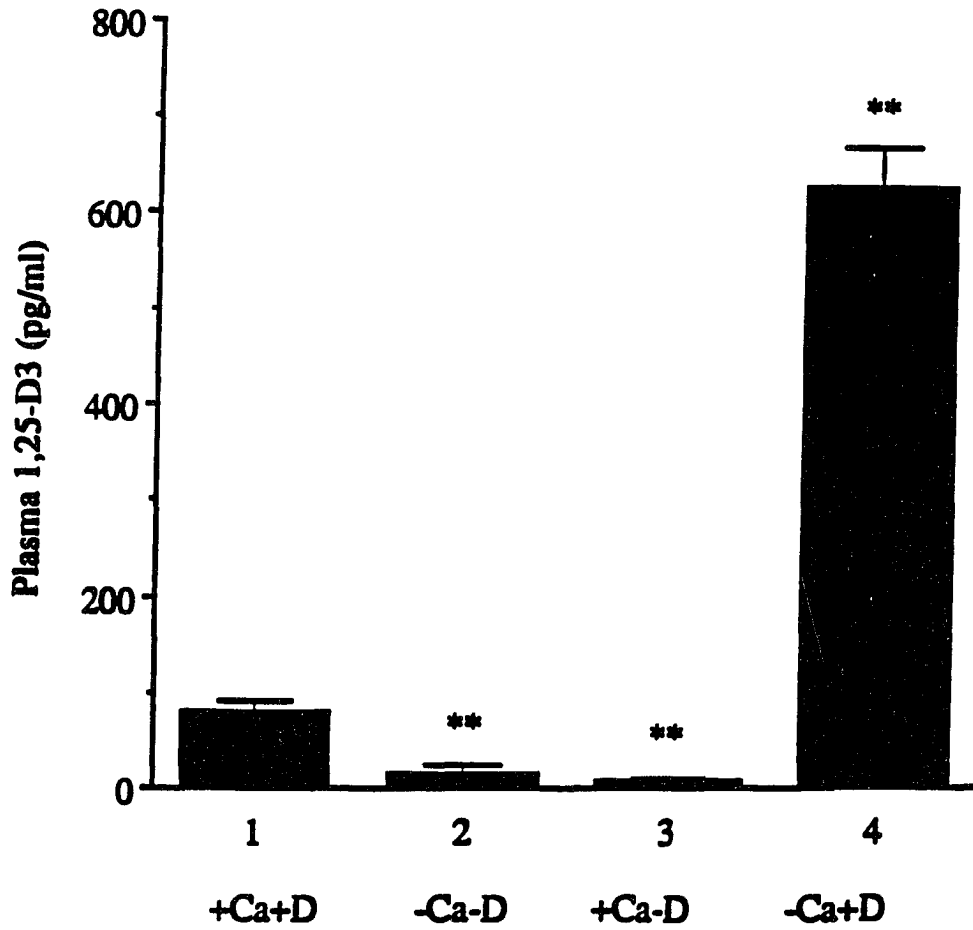


Figure 7. Plasma 1,25-D₃ concentration in rats fed different diets

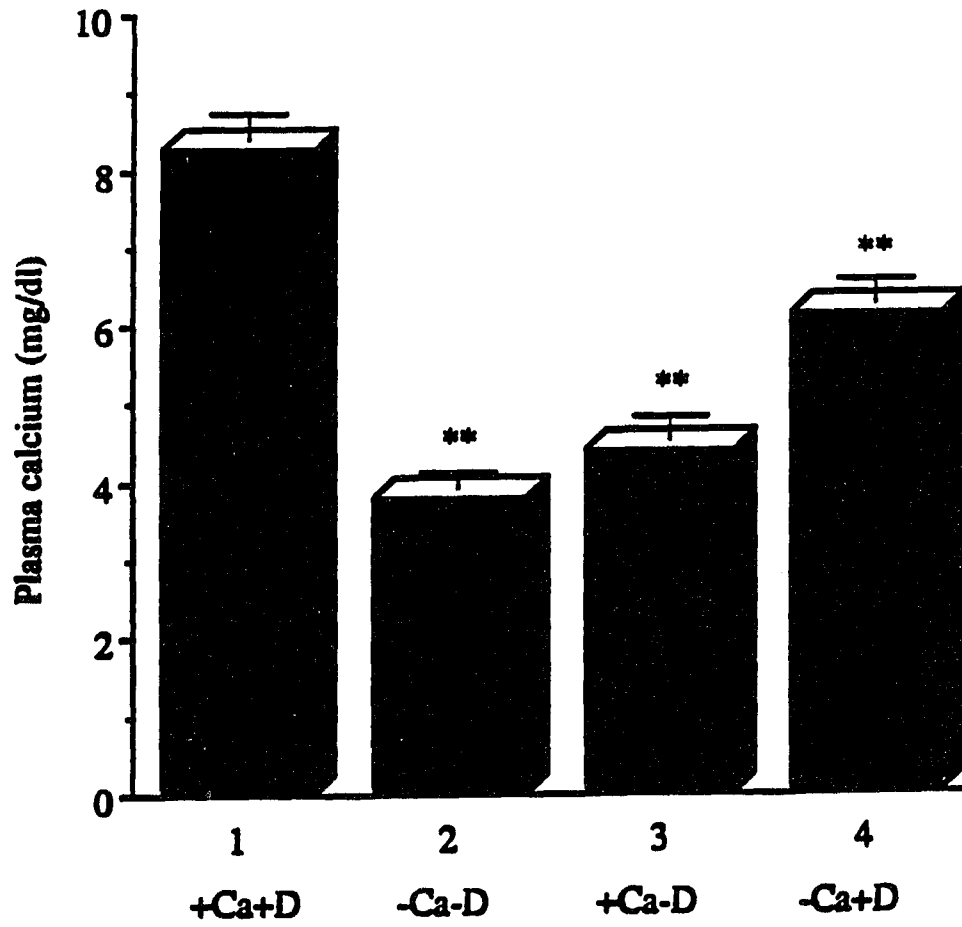


Figure 8. Plasma calcium concentration in rats fed different diets

Table 1. Intestinal CaATPase mRNA [relative value]

Dietary treatment	Mean	SD
+Ca+D	1.00	0
-Ca-D	0.50**	0.17
+Ca-D	0.59**	0.19
-Ca+D	0.84	0.13

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

Each group contains 4 samples.

Table 2. Intestinal CaBP mRNA [relative value]

Dietary treatment	Mean	SD
+Ca+D	1.00	0
-Ca-D	0.34**	0.26
+Ca-D	0.20**	0.07
-Ca+D	1.00	0.44

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

Each group contains 4 samples.

Table 3. Plasma 1,25-D3 concentration [pg/ml]

Dietary treatment	Mean	SD
+Ca+D	80.8	11.5
-Ca-D	15.6**	9.80
+Ca-D	7.80**	3.90
-Ca+D	622.4**	40.9

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

Each group contains 4 rat samples.

Table 4. Plasma calcium concentration [mg/dl]

Dietary treatment	Mean	SD
+Ca+D	8.30	0.30
-Ca-D	3.80**	0.20
+Ca-D	4.40**	0.30
-Ca+D	6.10**	0.30

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

Each group contains 4 rat samples.

**PREGNANCY AND LACTATION INCREASE
VITAMIN D-DEPENDENT INTESTINAL
MEMBRANE CALCIUM ATPase AND CALCIUM
BINDING PROTEIN mRNA EXPRESSION**

A paper to be submitted to the J. of Bone and Miner. Res.

Yingting Zhu, Jesse P. Goff, Timothy A. Reinhardt and Ronald L. Horst

Abstract

The calcium demands of pregnancy and lactation are known to upregulate intestinal calcium absorption. Intestinal epithelial cells contain calcium ATPases and calcium binding proteins, which are believed to play important roles in intestinal calcium transport. However, the possible role for these two proteins in the upregulation of intestinal calcium absorption observed in pregnancy and lactation is unknown. In this study, intestinal plasma membrane calcium ATPase [PMCA1], calcium binding protein [CaBP-9K] and vitamin D receptor [VDR] mRNA levels were determined by Northern blotting analysis at different stages of pregnancy and early lactation in rats. Intestinal calcium ATPase and calcium binding protein mRNA levels did not differ significantly among non-pregnant rats and rats

pregnant for 7 or 14 days. However, at 21 days gestation, both calcium ATPase and calcium binding protein mRNA levels increased 2- to 3-fold. Calcium ATPase and calcium binding protein mRNA remained elevated at 7 days of lactation. Plasma 1,25-dihydroxyvitamin D₃ [1,25-D₃] concentration exhibited a similar pattern, rising markedly at 21 days gestation and remaining elevated in lactation. Interestingly, VDR mRNA levels did not change during the entire experiment. But intestinal VDR content increased 2-fold in late pregnancy and lactation. These data suggest that the adaptation to higher requirement for calcium in late pregnancy and early lactation stimulates transcription of calcium absorption factors and the effects of gestation and lactation on VDR are probably post transcriptional.

Introduction

Intestinal calcium transport across the rat intestine can be transcellular or paracellular (6). The transcellular pathway is believed to function primarily in the small intestine. In contrast, the paracellular pathway can occur anywhere in the intestine (44), but functions only when calcium can passively diffuse down a concentration gradient from the

lumen to the blood. Transcellular calcium transport is an active process and is described as occurring in three steps: 1. calcium diffusion across the brush border membrane of the enterocyte; 2. translocation of calcium from the brush border to the basolateral membrane; and 3. active extrusion of calcium across the basolateral membrane against a concentration gradient (8, 60).

The entry of calcium into the cell can occur by simple diffusion across the plasma membrane down its electrochemical gradient since luminal calcium concentration is generally greater than intracellular calcium concentration. In addition, calcium may enter through voltage dependent or receptor operated channels coupled to second messengers, or through a $\text{Ca}^{2+}/\text{Na}^{+}$ exchanger (7). Once inside the cell, calcium moves to the basolateral membrane, either as free calcium ions, within vesicles, or bound to CaBP-9K (60). The concept of calbindin-9K as an intracellular calcium "carrier" is widely accepted. Intracellular calcium movement may be affected by other mobile organelles (37), but this transport is probably of relatively little importance. At the basolateral membrane, calcium ATPase plays a major role in extrusion of calcium (20). A $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger also is present but contributes comparatively little to calcium

extrusion (36).

The active metabolite of vitamin D, 1,25-D₃, has long been known to be the principal regulator of transcellular calcium absorption processes (3, 38, 52). It acts directly on the intestinal mucosal cells by a classical steroid hormone receptor-mediated process (13, 34) to induce the transcription and translation of proteins such as calcium binding protein (2, 3, 17, 33, 35, 39, 44, 52, 55, 61), CaATPase (15, 18, 42, 53, 57, 58) and vitamin D receptor (22, 47).

The active transport of calcium by the intestinal tract is increased when larger amounts of calcium are required during such physiological states as pregnancy and lactation (5, 19, 32, 56). The purpose of this study was to determine which components of the active calcium transport system are activated during pregnancy and early lactation.

Materials and methods

Animals: Three-month-old female non-pregnant, 7, 14, or 21 days pregnant and 7 days lactating rats fed rat lab diet [1% Ca, 0.74% P] (#5012, PMI Feeds, Inc., St. Louis, MO) were used to perform the experiment. Female rats were bred at weekly intervals so that all rats could

be euthanized on the same day. All pregnant rats had at least 10 fetuses and all lactation rats were nursing at least 10 pups. Rats were euthanized by inhalation of 50:50 CO₂:O₂ followed by decapitation. Blood samples were collected into heparinized tubes.

In one set of rats, the duodenum was removed for determination of VDR protein concentration by saturation binding analysis. In a second set of animals the proximal 15 cm of duodenum mucosa from each rat was obtained and frozen in liquid nitrogen for later mRNA analysis.

Northern blotting: Plasma membrane CaATPase, calcium binding protein and vitamin D receptor mRNA levels were determined by Northern blotting. Total RNA was isolated by TRIzol and chloroform, precipitated and washed by isopropanol and ethanol (GIBCO, BRL, Gaithersburg, MD). PolyA selection for detecting VDR mRNA was accomplished by streptavidin paramagnetic particles purchased from Promega, Madison, WI. RNA was fractionated on 1.2% formaldehyde agarose gel and transferred to nylon membranes (MSI Micro Separations Inc., Westboro, MA). The RNA on the membranes was cross-linked by UV Stratalinker 1800 (Stratagene, La Jolla, CA). A 3.4 kb cDNA from rat brain plasma membrane CaATPase [PMCA1], kindly supplied by Dr. Gary

Shull, University of Cincinnati, OH, was used to hybridize the membranes for analysis of CaATPase mRNA. The cDNA for CaBP-9K mRNA assay was the gift of Dr. M. Elizabeth Bruns, University of Virginia Medical School, Charlottesville, VA. The VDR cDNA was provided by Dr. Wes Pike, Ligand Pharmaceutical, La Jolla, CA.

Membranes were prehybridized at 42^o C for 4-6 hours in a prehybridization buffer containing 50% formamide, 5X Denhardt's reagent, 0.1% sodium dodecyl sulfate [SDS] and 100 ug/ml denatured fish sperm DNA. Fresh prehybridization buffer and 250 ug tRNA was used for the overnight membrane hybridization with 5 x 10⁶ cpm ³²P-labeled cDNA probe/ml prehybridization buffer. The membranes were washed twice in 2 X SSPE/0.1% SDS buffer [SSPE is NaCl, NaH₂PO₄, EDTA mixture], twice in 0.1X SSPE/0.1% SDS, each for ten minutes.

Quantitative analysis of mRNA was done by Electronic Autoradiography (PACKARD, Meriden, CT). A 2.1 kb chick β-actin cDNA probe was obtained by digestion of pBR322 with Hind III (12) and used as a control for estimation of mRNA integrity and RNA sample loading difference among various preparations.

Unoccupied VDR assay: Intestinal tissues were collected and mucosa

was harvested. Mucosa was washed 3 times in buffer [10 mM Tris, 1.5 mM ethylenediaminetetraacetic acid, 2 mM dithiothreitol and 200 ug/ml soybean trypsin inhibitor] with low speed centrifugation between each washing step (Costa and Feldman, 1986). A 20% homogenate [w/v] of the mucosa was prepared with a polytron (Brinkmann, Des Plaines, IL) in buffer containing 600 mM KCl. The homogenate was centrifuged at 150,000 xg for 20 minutes to obtain cell cytosol. Unoccupied VDR was estimated by incubating the cell cytosol with 3.6 nM 1,25-D₃ at 4^o C for 18 hours with gently shaking. Receptor bound 1,25-[26,27-³H]D₃ was determined with hydroxyapatite. Specific binding was obtained by the difference between total binding and that observed in the presence of excess radio-inert 1,25-D₃. Results are expressed in fmoles 1,25-D₃ bound/mg cytosol protein [fmole/mg].

Plasma 1,25-D₃ determination: The method of Reinhardt et al (45) was modified to determine 1,25-D₃ concentration in plasma (45). The plasma 1,25-D₃ was chromatographed on C18/OH low hydrocarbon columns (Varian, Harbor City, CA), and the concentration determined by radio-receptor assay. Recovery of [³H]1,25-D₃ was used to estimate recovery of 1,25-D₃ through the purification steps for each sample.

Plasma calcium concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer Corporation. 1965 Analytical methods for atomic absorption spectrophotometry. Perkin-Elmer Corp., Norwalk, CT).

Analysis of variance was used to determine if differences among CaATPase, CaBP and VDR mRNA levels and plasma constituents existed as a result of reproductive state. When ANOVA suggested differences existed, Dunnett multiple comparisons test was used to test the significance between individual stages of gestation.

Results

Intestinal CaATPase mRNA expression in the duodenum from 21-day pregnant and 7-day lactation rats was increased 2.6- and 3.2-fold respectively above that in non-pregnant rats [$p < 0.01$] (Figure 1, 2 and Table 1). Intestinal CaBP-9K mRNA expression in the duodenum of 21-day pregnant and 7-day lactation rats was increased 2.1- and 3.2-fold above non-pregnant rats [$p < 0.05$ and 0.01], respectively, for CaBP mRNA (Figure 3, 4 and Table 2). Northern blots of CaATPase exhibited a 3-band pattern of hybridization with a major band corresponding to 6 kb, which

is similar to previous reports (Figure 2) (37). There was no significant increase in CaATPase or CaBP mRNA at 7 or 14 days of gestation. Intestinal VDR mRNA did not change at any time during the entire period of gestation and lactation (Figure 5, 6 and Table 3). However, intestinal VDR content increased significantly in late pregnancy and lactation [$p < 0.05$] (Figure 7 and Table 4). Peak plasma 1,25-D₃ elevation was at 21 days of pregnancy, increasing from the control level of 35.6 pg/ml to 165.5 pg/ml [$p < 0.001$], and remained high during lactation [$p < 0.01$] (Figure 8 and Table 5). Plasma calcium concentration decreased from 11.2 mg% in non-pregnant rats to 8.0 mg% in 21-day pregnancy [$p < 0.01$], and remained low at the 7th day of lactation (Figure 9 and Table 6).

Discussion

Active transport of calcium across the intestinal epithelial cells is dependent on the active metabolite of vitamin D, 1,25-D₃ (14, 60, 63). The increased concentrations of plasma 1,25-D₃ during late pregnancy and early lactation allows the dam to adapt to the large calcium demands of the fetus and lactation. Other factors such as estrogen may be involved because an increase in calcium transport rate in vitamin D-deficient pregnant rats

has also been observed (5). However, this increase was significantly lower than in vitamin D-repleted pregnant rats (5, 27). The fetus accumulates most of its body calcium over the last one third of gestation (21), and loss of calcium to milk during lactation represents an even greater calcium demand. Increased concentrations of plasma 1,25-D₃ during late pregnancy and early lactation have been reported in rats and human (28, 43, 62). Interestingly, pregnancy evidently does not change the circulating levels of 1,25-D₃ in the sheep (41). Our data indicate a 3- to 4-fold increase of plasma 1,25-D₃ during late pregnancy and early lactation, which is in agreement with earlier reports (28, 43).

The calcium binding proteins [CaBP-9K], present in mammalian intestine, and CaBP-28K, in mammalian kidney, avian kidney and intestine are the most extensively studied products of genes affected by 1,25-D₃ (4, 11, 16, 24, 31, 51, 54). The induction of these gene products generally coincides with increased calcium transport (25, 59). The rat placental CaBP-9K was reported to be upregulated during late pregnancy (21). Previous reports have suggested that intestinal CaBP-9K mRNA was upregulated only in early lactation and not in pregnancy (30). CaATPase extrudes calcium from the basolateral membrane of intestinal cells to the

extracellular fluids (9, 48). At least four genes have been found to code for plasma membrane CaATPase (9, 29). These isoforms of plasma membrane CaATPase [PMCA] include PMCA1, PMCA2, PMCA3 and PMCA4. All four isoforms of PMCA and their mRNAs have been identified in rats (23, 29, 49). PMCA1 mRNA was expressed in virtually all tissues, whereas other isoforms of PMCA were tissue-specific (23). In rat and human intestine, PMCA1 is the predominant CaATPase isoform (23, 29) and is regulated by vitamin D (54, 63). A rat placental CaATPase mRNA was also shown to be upregulated during late pregnancy and early lactation (21); but little is known of changes in rat intestinal CaATPase mRNA expression during late pregnancy and early lactation.

Interestingly, our results indicated that VDR mRNA did not change during pregnancy and lactation. However, unoccupied VDR number nearly doubled during late pregnancy and lactation. This suggested that the effects of pregnancy and lactation on VDR appears to change VDR metabolism by post transcriptional and nongenomic mechanisms that are consistent with reports of prolongation of the half life of the receptor in the occupied state (1, 50).

Our studies demonstrate a 2-3 fold increase in both vitamin D-

dependent intestinal CaATPase and CaBP-9K mRNA in 21-day pregnant and 7-day lactation rats. The increased expression of calcium ATPase and calcium binding protein mRNA implies that active transport systems of calcium in the intestine are activated when a sudden, large amount of calcium is required, such as during late pregnancy when the fetus accumulates calcium in bones and other soft tissues and early lactation of animals when large amounts of calcium are "lost" in milk. CaBP and CaATPase activities may be enhanced to compensate for physiological losses of large amount of calcium during late pregnancy and early lactation.

Acknowledgment

The authors thank Dr. Richard L. Engen, Dr. Franklin A. Ahrens, Dr. Donald C. Beitz for all their support of the project. The authors also thank Derrel A. Hoy, Judy M. Pochop, Cynthia A. Hauber, K. C. Hummel, Mary Kassinos and Gary Fry for their technical support.

References cited

- 1. Arbour, N. C., Prah, J. M. & DeLuca, H. F. 1993. Stabilization of the vitamin D receptor in rat osteosarcoma cells through the action of 1,25-dihydroxyvitamin D₃. *Mol. Endocrinol.* 7:1307-1312.**
- 2. Bar, A., & Wasserman, R. H. 1973. Control of calcium absorption and intestinal calcium binding protein synthesis. *Biochem. Biophys. Res. Commun.* 54:191-196.**
- 3. Bar, A. & Hurwitz, S. 1979. *Endocrinology*. The interaction between dietary calcium and gonadal hormones in their effect on plasma calcium, bone, 25-hydroxycholecalciferol-1-hydroxylase, and duodenal calcium-binding protein, measured by a radioimmunoassay in chick. 104:1455-1460.**
- 4. Brehier, A. & Thomasset, M. 1990. Stimulation of calbindin-D9K (CaBP9K) gene expression by calcium and 1,25-dihydroxyvitamin D₃ in fetal duodenal organ culture. *Endocrinology*. 127:580-587.**
- 5. Boass, A., Lovdal, J. A. & Toverud, S. U. 1992. Pregnancy- and lactation-induced changes in active intestinal calcium transport in rats. *Am. J. Physiol.* 262:G127-G134.**
- 6. Bronner, F., Pansu, D. & Stein, W. D. 1986. An analysis of intestinal calcium transport across the rat intestine. *J. Physiol.* 250:G565-G569.**
- 7. Bronner, F. 1991. Calcium transport across epithelia. *Int. Rev. Cytol.* 131:169-212.**
- 8. Bronner, F. 1992. Current concepts of calcium absorption; an overview. *J. Nutr.* 122:641-643.**
- 9. Carafoli, E. 1987. Intracellular calcium homeostasis. *Annu. Rev. Biochem.* 56:395-433.**

10. Carafoli, E. 1992. The calcium pump of plasma membrane. *J. Biol. Chem.* 267:2115-2118.
11. Christakos, S., Gabrielides, C. & Rhoten, W. B. 1989. Vitamin D-dependent calcium binding proteins: chemistry, distribution, functional consideration, and molecular biology. *Endocrinol. Rev.* 10:3-16.
12. Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J. & Kirschner, M. W. 1980. Number and evolutionary conservation of a α and β tubulin and cytoplasmic β and γ actin genes using specific cloned cDNA probes. *Cell.* 20:95-105.
13. DeLuca, H. F & Schnoes, H. K. 1976. Metabolism and mechanism of action of vitamin D. *Annu. Rev. Biochem.* 45:631-666.
14. Favus, M. J. 1985. Factors that influence absorption and secretion of calcium in the small intestine and colon. *Am. J. Physiol.* 248:G147-157.
15. Favus, M. J., Tenbe, V., Ambrosic, K. A. & Nellans, H. N. 1989. Effects of 1,25-dihydroxyvitamin D₃ on enterocyte basolateral membrane calcium transport in rats. *Am. J. Physiol.* 256:G613-G617.
16. Ferrari, S., Molinari, S., Battini, R., Cossu, G. & Lamon, Fava S. 1992. Induction of calbindin-D28K by 1,25-dihydroxyvitamin D₃ in cultured chicken intestinal cell. *Exp. Cell Res.* 200:528-531.
17. Fox, J., Pickard, D. W., Care, D. & Murray, T. M. 1978. Effect of low phosphorus diet on intestinal calcium absorption and the concentration of calcium-binding protein in intact and parathyroidectomized pigs. *Endocrinology.* 78:379-387.
18. Freedman, R. A., Weiser, M. M., & Isslbacher, K. J. 1977. Calcium transport by Golgi and lateral membrane vesicles from rat intestine; decrease in vitamin D-deficient rats. *Proc. Natl. Acad. Sci. USA* 74:3612-3616.
19. Gertner, J. M., Coustan, D. R., Kligler, A. S., Mallette, L. E., Ravin, N. and Broadus, A. E. 1986. Pregnancy as state of

physiologic absorptive hypercalciuria. *Am. J. Med.* 81:451-456.

20. Ghijssen, W. E., DeJong, M. D. & van Os, C. H. 1982. ATP-dependent calcium transport and its correlation with Ca^{2+} -ATPase activity in basolateral plasma membranes of rat duodenum. *Biochim. Biophys. Acta* 689:327-336.

21. Glazier, J. D., Atkinson, D. E., Thornburg, K. L., Sharpe, P. T., Edwards, D., Boyd, R. D. H. & Sibley, C. P. 1992. Gestational changes in Ca^{2+} transport across rat placenta and mRNA for calbindin-9K and $\text{Ca}^{(2+)}$ -ATPase. *Am. J. Physiol.* 262:R930-R935.

22. Goff, J. P., Horst, R. L. & Reinhardt, T. A. 1989. Effects of continuous administration of 1,25-dihydroxyvitamin D_3 on plasma minerals and unoccupied colon mucosal 1,25-dihydroxyvitamin D receptor concentrations. *J. Dairy Sci.* 72:2936-2941.

23. Greeb, J. & Shull, G. E. 1989. Molecular cloning of a third isoform of the calmodulin-sensitive plasma membrane Ca^{2+} transporting ATPase that is expressed predominantly in brain and skeletal muscle. *J. Biol. Chem.* 264:18569-18576.

24. Gross, M. D. & Kumar, R. 1990. Vitamin D metabolism and mechanisms of calcium transport. *J. Am. Soc. Nephrol.* 1:30-42.

25. Gross, M. D. & Kumar, R. 1990. Physiology and biochemistry of vitamin D-dependent calcium binding proteins. *Am. J. Physiol.* 259:F195-F209.

26. Grover, A. K. & Khan, I. 1992. Calcium pump isoforms: diversity, selectivity, and plasticity. Review article. *Cell Calcium.* 13:9-17.

27. Halloran, B. P. & DeLuca, H. F. 1980. Calcium transport in small intestine during pregnancy and lactation. *Am. J. Physiol.* 239:E64-E68.

- 28. Horst, R. L. & Reinhardt, T. A.** 1988. Vitamin D molecular, cellular and clinical endocrinology. Walter de Gruyter & Co., Berlin. Newyork-Printed in Germany.
- 29. Howard, A., Legon, S. & Walters, J. F.** 1993. Human and rat intestinal plasma membrane calcium pump isoforms. *Am. J. Physiol.* 263:G917-G925.
- 30. Krisinger, J., Dann, J. L., Jeung, E. B. & Leung, P. C. K.** 1992. Calbindin-D9k gene expression during pregnancy and lactation in rat. *Molecular and cellular Endocrinology.* 88:119-128.
- 31. Kumar, R.** 1990. Vitamin D and mechanisms of calcium transport. *J. Am. Soc. Nephrol.* 1: 30-42.
- 32. Lobaugh, B., Boass, A., Lester, G. E. & Toverud, S. U.** 1990. Regulation of serum 1,25-dihydroxyvitamin D₃ in lactating rats. *Am. J. Physiol.* 259:E665-E671.
- 33. Meyer, J., Fullmer, C. S., Wasserman, R. H., Komm, B. S. & Haussler, M. R.** 1992. Dietary restriction of calcium, phosphorus and vitamin D elicits differential regulation of the mRNAs for avian intestinal calbindin-D28K and the 1,25-dihydroxyvitamin D receptor. *J. Bone Miner. Res.* 7:441-448.
- 34. Minghetti, P. P. & Norman, A. W.** 1988. 1,25-dihydroxyvitamin D receptors: gene regulation and genetic circuitry. *FASEB J.* 2:3043-3053.
- 35. Morrissey, R. L., & Wasserman, R. H.** 1971. Calcium absorption and calcium-binding protein in chicks on different calcium and phosphate intakes. *Am. J. Physiol.* 220:1509-1515.
- 36. Nellans, H. N. & Popovitch, J. R.** 1984. Role of sodium in intestinal calcium transport. In: *Epithelial calcium and phosphate transport: molecular and cellular aspects.* (Bronner, F. and Peterlik, M. eds), pp 301-306, AlanR. Liss, New York, NY.

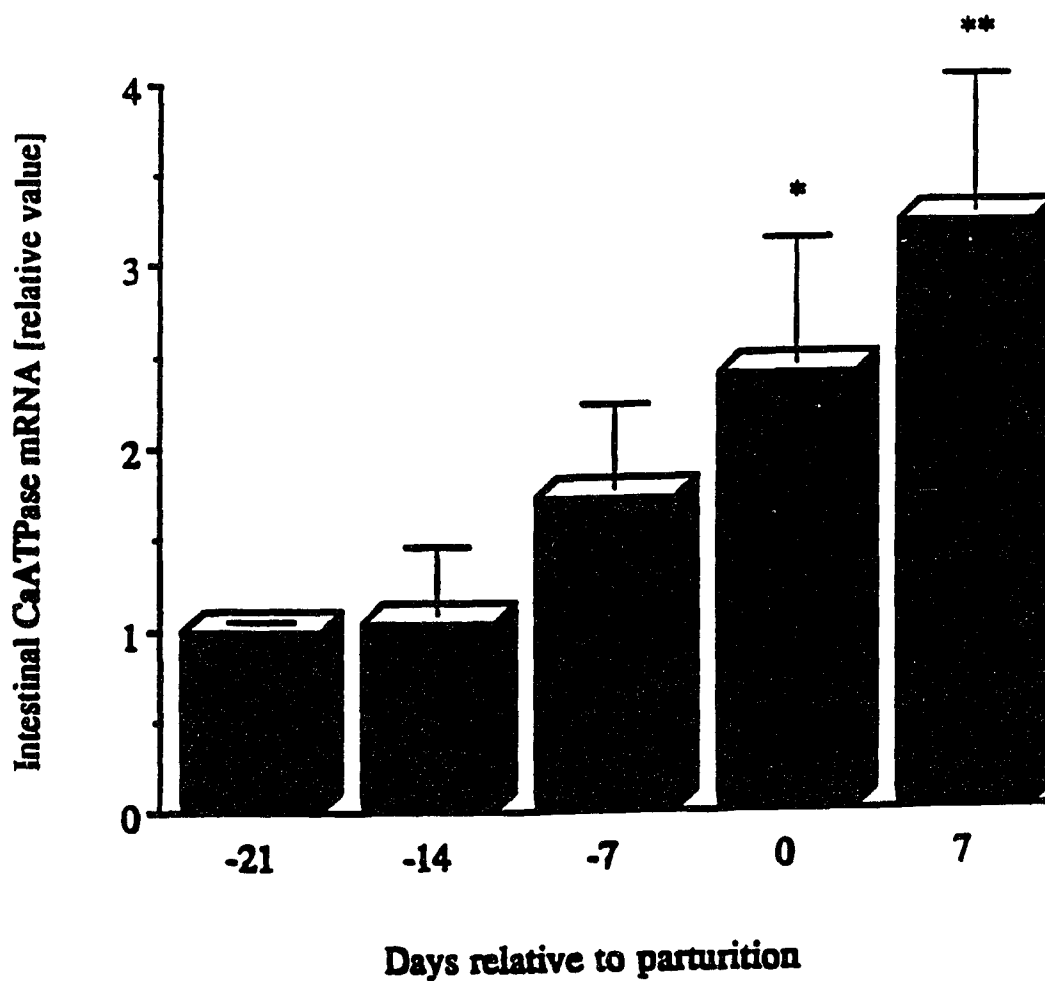
- 37. Nemere, I.** 1990. Organelles that bind calcium. In: *Intracellular calcium regulation*. (Bronner, F., ed) pp. 163-179, Wiley-Liss New York, NY.
- 38. Norman, A. W.** 1984. Hormonal actions of vitamin D. *Curr. Top. Cell Regul.* 24:35-49.
- 39. Pansu, D., Ballaton, C., & Bronner F.** 1981. Effect of Ca intake on saturable and nonsaturable components of duodenal Ca transport. *Am. J. Physiol.* 240:G32-G37.
- 40. Pansu, D., Bellaton, C., Roche, C. & Bronner, F.** 1983. Duodenal and ileal calcium absorption in the rat and effects of vitamin D. *Am. J. Physiol.* 244:G695-G700.
- 41. Paulson, S. K. & Langman, C. B.** 1990. Plasma vitamin D metabolite levels in pregnant and non-pregnant ewes. *Comp. Biochem. Physiol. A.* 96:347-349.
- 42. Qiang Cai, John, S. Chandler, Robert H. Wasserman, Rajiv Kumar & John T. Penniston** 1993. Vitamin D and adaptation to dietary calcium and phosphate deficiencies increase intestinal plasma membrane calcium pump gene expression. *Proc. Natl. Acad. Sci.* 90:1345-1349.
- 43. Quan, S. D. & Miller, S. C.** 1989. Calcitropic hormone levels and calcium absorption during pregnancy in rats. *Am. J. Physiol.* 257:E118-E123.
- 44. Rader, J. I., Baylink, D. J., Hughes, M., Safilian, E. F., & Haussler, M. L.** 1979. Calcium and phosphorus deficiency in rats. effects on PTH and 1,25-dihydroxyvitamin D₃. *Am. J. Physiol.* 236, E118-E122.
- 45. Reinhardt, T. A., Horst, R. L., Orf, J. W. & Hollis, B. W.** 1984. A microassay for 1,25-dihydroxyvitamin D₃ not requiring high performance liquid chromatography: application to clinical studies. *J. Clin. Endocrinol. Metab.* 59:91-98.

- 46. Reinhardt, T. A. & Horst, R. L. 1990.** Parathyroid hormone down-regulates 1,25-dihydroxyvitamin D receptor (VDR) and VDR messenger ribonucleic acid in vitro and blocks homologous upregulation of VDR in vivo. *Endocrinology*.127:942-948.
- 47. Reinhardt, T. A., Horst, R. L. & Goff, J. P. 1993.** 1,25-dihydroxyvitamin D receptor occupancy is limited: dissociation of occupied receptor levels from plasma 1,25-dihydroxyvitamin D levels. *Endo. J.* 1:317-322.
- 48. Schatzmann, H. J. 1989.** The calcium pump of the surface membrane and of the sarcoplasmic reticulum. *Annual Rev. Physiol.* 51:473-485.
- 49. Shull, G. E. & Greeb, J. 1988.** Molecular cloning of two isoforms of the plasma membrane Ca^{2+} -transporting ATPase from rat brain. *J. Biol. Chem.* 263:8646-8657.
- 50. Sriussadaporn, S., Wong, M. S., Pike, J. W. & Favus, M. J. 1995.** Tissue specificity and mechanism of vitamin D receptor up-regulation during dietary phosphorus restriction in the rat. *J. Bone Miner. Res.* 10:271-280.
- 51. Strom, M., Krisinger, J., Darwish, H. & Deluca, H. F. 1992.** 1,25-dihydroxyvitamin D and not calcium is the major regulator of calbindin-D 9-kDa mRNA levels in vivo. *Proc. Soc. Exp. Biol. Med.* 199:369-371.
- 52. Swaminathan, R., Sommerville, B. A., & Care A. D. 1977.** The effect of dietary calcium on the activity of 25-hydroxycholecalciferol-1-hydroxylase and Ca absorption in vitamin D-replete chicks. *Br. J. Nutr.* 38:47-54.
- 53. Takito, J., Shinki, T., Sasaki, T. & Suda, T. 1990.** Calcium uptake by brush-border and basolateral membrane vesicles in chick duodenum. *Am J. Physiol.* 258:G16-G23.

- 54. Takito, J., Shinki, T., Tanaka, H. & Suda, T.** 1992. Mechanism of regulation of calcium pumping activity in chick intestine. *Am. J. Physiol.* 262:G797-G805.
- 55. Thomasset, M., Cuisinier-Gleizes, P. & Mathieu, H.** 1977. Differences in duodenal calcium-binding protein (CaBP) in response to a low-calcium or low-phosphorus intake. *Calcif. Tiss. Res.* 22S:45-50.
- 56. Toverud, S. U., Boass, A., Haussler, M. R. & Pike, J. W.** 1983. Circulating levels and function of 1,25-dihydroxyvitamin D₃ in lactation. *J. Steroid Biochem.* 19:505-510.
- 57. Van Os, C. H.** 1987. Transcellular calcium transport in intestine and renal epithelial cells. *Biochim. Biophys. Acta.* 906:195-222.
- 58. Walters, J. R. F. & Weiser, M. M.** 1987. Calcium transport by rat duodenal villus and crypt basolateral membranes. *Am. J. Physiol.* 252:G170-G177.
- 59. Wasserman, R. H.** 1981. Intestinal absorption of calcium and phosphorus. *Fed. Proc.* 40:68-72.
- 60. Wasserman, R. H., Chandler, J. S., Meyer, S. A., Smith, C. A., Brindak, M. E., Fullmer, C. S., Penniston, J. T. & Kumar R.** 1992. Intestinal calcium transport and calcium extrusion processes at basolateral membrane. *J. Nutr.* 122:662-671.
- 61. Wasserman, R. H.** 1992. Extra and intracellular calcium and phosphate regulation. eds. Bronner, F. & Peterlik, M. (CRC, Boca Raton, FL, Cha. 5).
- 62. Wilson, S. G., Retallack, R. W., Kent, J. C., Worth, G. K. & Gutteridge, D. H.** 1990. Serum free 1,25-dihydroxyvitamin D and free 1,25-dihydroxyvitamin D index during longitudinal study of pregnancy and lactation. *Clin. Endocrinol. Oxf.* 32:613-622.

63. Zelinski, J. M., Sykes, D. E. & Weiser, M. M. 1991. The effect of vitamin D on rat intestinal membrane Ca-pump mRNA. *Biochem. Biophys. Res. Commun.* 179:749-755.

Appendix with mean data tables



**Figure 1. Intestinal CaATPase mRNA expression
in pregnancy and lactation**

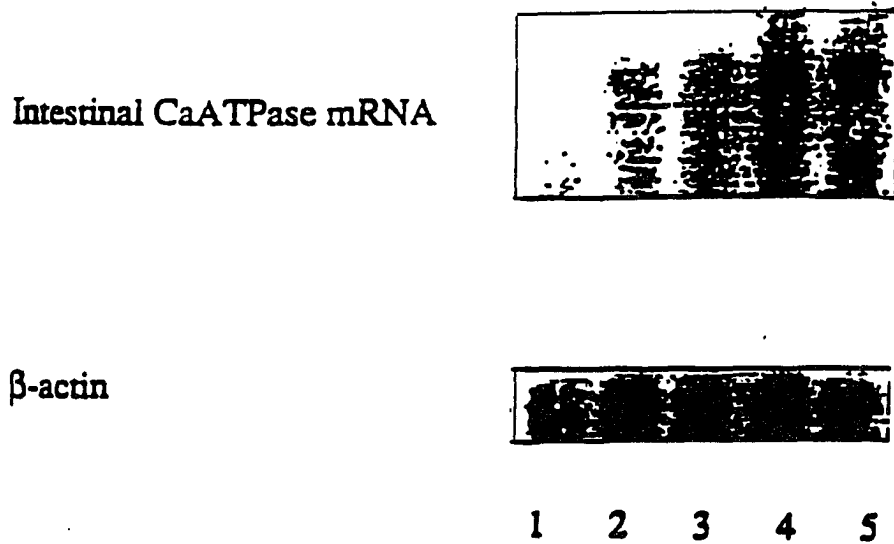
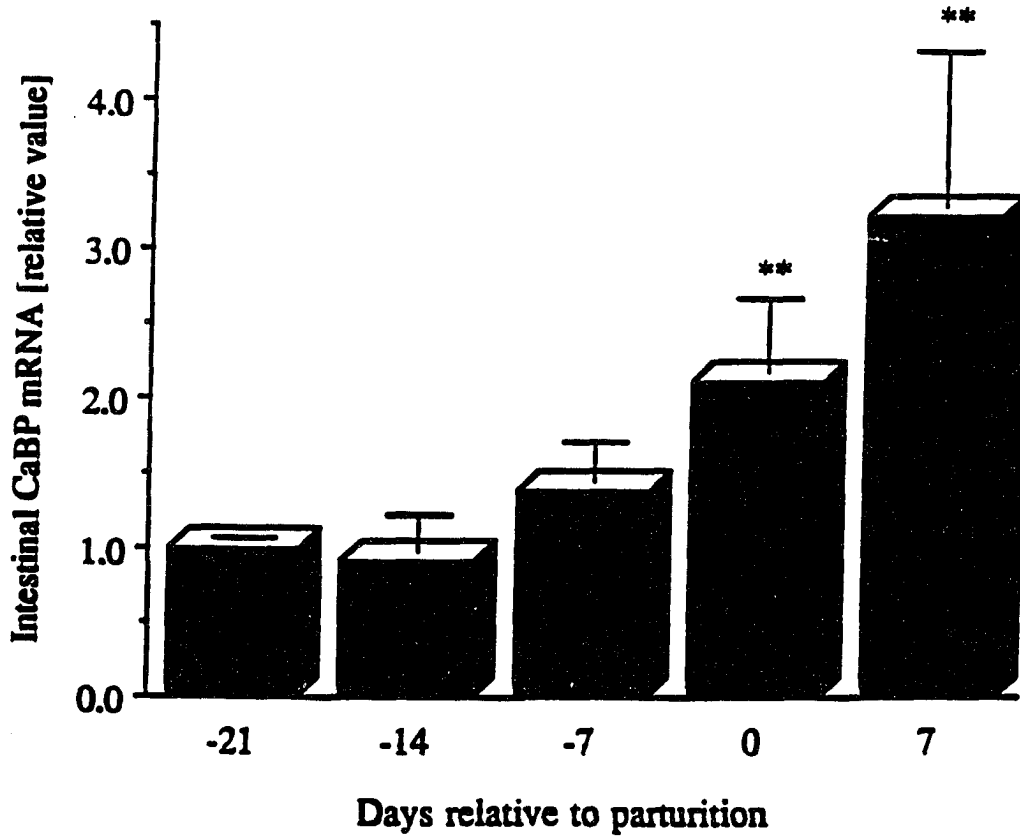


Figure 2. Northern blot of intestinal CaATPase mRNA

1. Non-pregnancy, non-lactation
2. 7-day pregnancy
3. 14-day pregnancy
4. 21-day pregnancy
5. 7-day lactation



**Figure 3. Intestinal CaBP mRNA expression
in pregnancy and lactation**

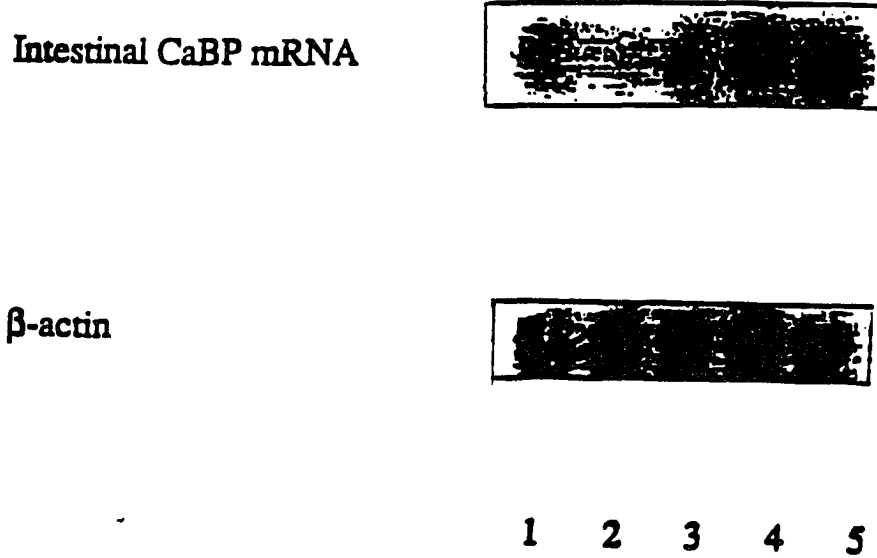
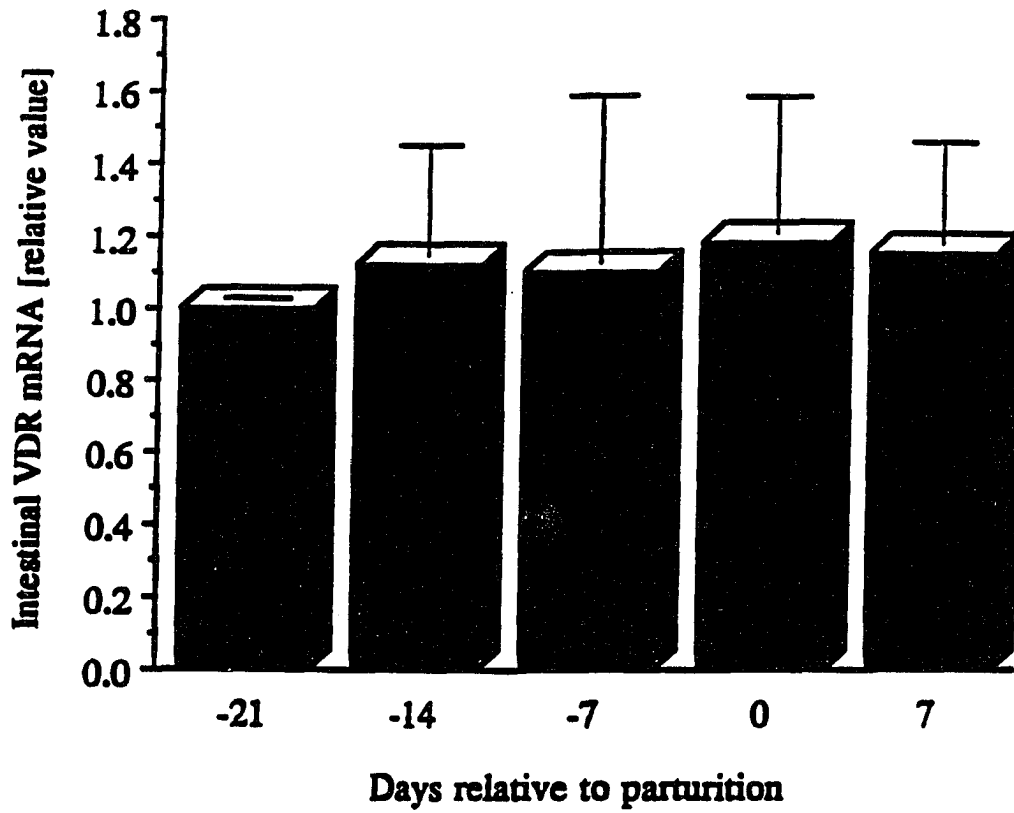


Figure 4. Northern blot of intestinal CaBP mRNA

- 1. Non-pregnancy, non-lactation**
- 2. 7-day pregnancy**
- 3. 14-day pregnancy**
- 4. 21-day pregnancy**
- 5. 7-day lactation**



**Figure 5. Intestinal VDR mRNA expression
in pregnancy and lactation**

Intestinal VDR mRNA

 β -actin

1 2 3 4 5

Figure 6. Northern blot of intestinal VDR mRNA

- 1. Non-pregnancy, non-lactation**
- 2. 7-day pregnancy**
- 3. 14-day pregnancy**
- 4. 21-day pregnancy**
- 5. 7-day lactation**

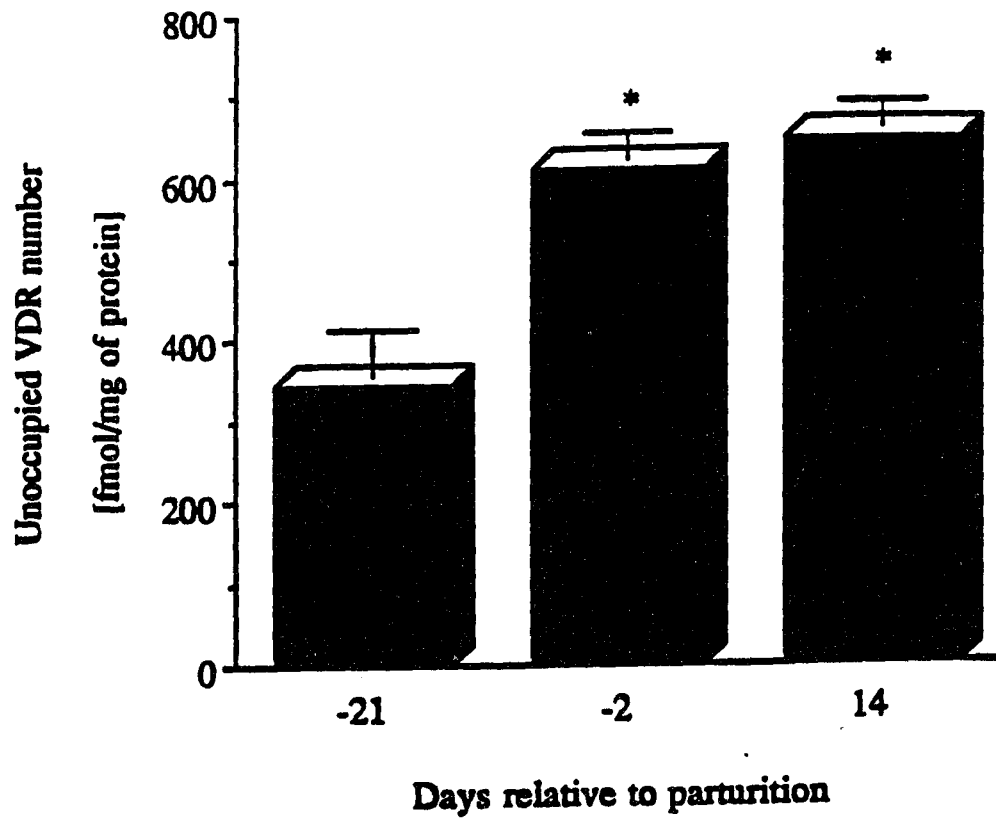


Figure 7. Intestinal VDR number in pregnancy and lactation

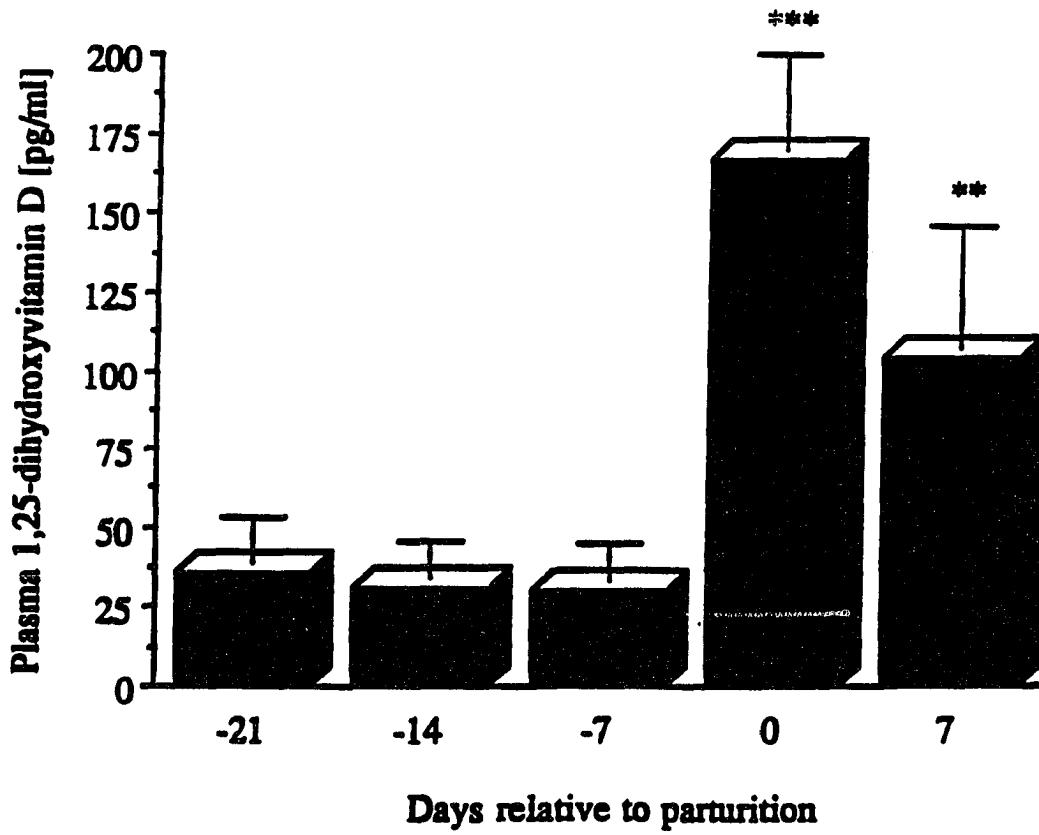


Figure 8. Plasma 1,25-D₃ concentration in pregnancy and lactation

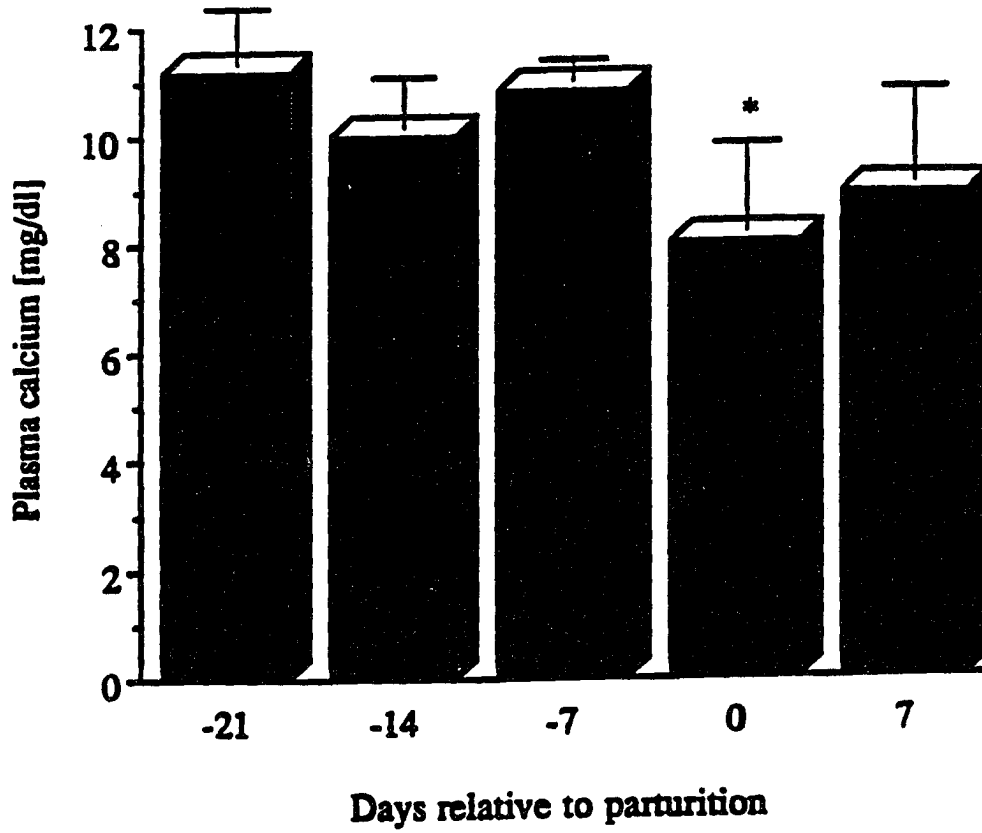


Figure 9. Plasma calcium concentration in pregnancy and lactation

Table 1. Intestinal CaATPase mRNA [relative value]

Days relative to parturition	Mean	SD
-21	1.00	0
-14	1.03	0.37
-7	1.72	0.45
0	2.39*	0.67
7	3.21**	0.75

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

Each group contains 4 rat samples.

Table 2. Intestinal CaBP mRNA [relative value]

Days relative to parturition	Mean	SD
-21	1.00	0
-14	0.91	0.25
-7	1.38	0.25
0	2.11**	0.47
7	3.21**	1.03

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

Each group contains 4 rat samples.

Table 3. Intestinal VDR mRNA [relative value]

Days relative to parturition	Mean	SD
-21	1.00	0
-14	1.12	0.30
-7	1.10	0.46
0	1.18	0.37
7	1.15	0.28

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

Each group contains 4 rat samples.

Table 4. Intestinal VDR number
[fmol/mg of protein]

Days relative to parturition	Mean	SD
-21	343	57
-2	610*	30
14	645*	32

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

Each group contains 4 rat samples.

Table 5. Plasma 1,25-D3 concentration [pg/ml]

Days relative to parturition	Mean	SD
-21	35.6	14.1
-14	30.9	11.6
-7	29.8	11.8
0	165.5***	30.8
7	103.8**	38.4

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

*** $p < 0.001$ compared to control group

Each group contains 4 rat samples.

Table 6. Plasma calcium concentration [mg/dl]

Days relative to parturition	Mean	SD
-21	11.2	1.0
-14	10.0	0.9
-7	10.8	0.4
0	8.0*	1.6
7	8.9	1.7

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

Each group contains 4 rat samples.

GENERAL CONCLUSIONS

1. Administration of 1,25-D₃ to vitamin D-deficient rats yielded a time-dependent upregulation of intestinal CaATPase and CaBP mRNA. We conclude that vitamin D is a major regulator of intestinal calcium transporting factors.

2. Large doses of 1,25,28-D₂ may induce CaATPase and CaBP mRNA. The results indicate that larger doses of 1,25-D₃ analog, 1,25,28-D₂, can also enhance transcriptional regulation of CaATPase and CaBP.

3. Vitamin D-deficiency downregulates CaATPase and CaBP mRNA. Calcium deficiency did not induce transcriptional upregulation of CaATPase and CaBP in our experiments. We suggest that vitamin D, not calcium, is a major transcriptional regulator of CaATPase and CaBP.

4. Pregnancy and lactation stimulate intestinal CaATPase and CaBP expression, implying the active transport systems are activated during late pregnancy and early lactation of animals.

5. Intestinal VDR mRNA did not increase during late pregnancy and early lactation. However, intestinal VDR numbers increase significantly. This observation suggests post transcriptional regulation of VDR.