Distribution and properties of Ca^{2+} -ATPase, phytase, and alkaline phosphatase in isolated enterocytes from normal and vitamin D-deficient rats

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SUMMARY The effects of vitamin D-deficiency and repletion on the distribution and activities of Ca^{2+} -ATPase, phytase, and alkaline phosphatase in intact epithelial cells isolated from different regions of the villi and the crypts of the rat jejunum were studied. Similar distribution patterns of activities were found for the three enzymes. In all cases, the enzyme levels were the highest at the villus tip and gradually declined to low activities in the crypt. The Kms were very different between cells in the crypt base and those at the villus tip, the highest Kms being found in the crypt. The activities of these enzymes were reduced in the entire length of the villus in vitamin D-deficient rats. Recovery of the enzymatic levels was observed on vitamin D repletion, but at different rates. Total recovery of activity of Ca^{2+} -ATPase, phytase, and alkaline phosphatase was observed after 18, 24, and 36 hours, respectively, after a single dose of 6.5 nmol (2.5 μg) vitamin D_3 . Enzymatic activities in the crypt cells were not affected by vitamin D_3 treatment. These data suggest that Ca^{2+} -ATPase, phytase, and alkaline phosphatase may be distinct entities, and that their activities in the crypt cells may not be vitamin D-dependent.

Vitamin D_3 is an essential regulator of calcium metabolism. It is now clear that vitamin D_3 represents a precursor for a steroid hormone-like metabolite, 1,25-(OH) $_2\text{D}_3$. This metabolite appears to mediate the biological actions of the vitamin, namely the control of skeletal development and the intestinal absorption of calcium and phosphorus. Although steroid hormone-like receptors have been demonstrated in the small intestinal mucosa,¹ it is still not clear how the effects of the vitamin are exerted at the molecular level.

A number of biochemical changes have been observed in chick small intestinal mucosal cells in response to vitamin D_3 . These include induced synthesis of calcium-binding protein,² increased enzymatic levels of Ca^{2+} -ATPase,³ phytase,⁴ alkaline phosphatase,⁵ increased synthesis of a number of proteins, one of which is actin-like⁶ and an increased turnover of the fatty acids in phospholipid side chains in the brush border membrane.⁷

The dependence of Ca^{2+} -ATPase (EC 3.6.1.3) in

the brush border of chick intestine upon vitamin D_3 has been recognised since the last decade^{3,8} and a role for this enzyme in Ca^{2+} transport in small intestinal cells has been proposed. There is evidence showing that Ca^{2+} -ATPase is stimulated at the same time as Ca^{2+} transport in response to 1,25-(OH) $_2\text{D}_3$ treatment.⁹

Increased activity of intestinal phytase (EC 3.1.3.8) of rachitic rats and chicks was noted on vitamin D-repletion.⁴ No clear role has been documented for this enzyme. It was proposed that phytase activity is associated with the same protein as alkaline phosphatase¹⁰ and that it functions by hydrolysing phosphate residues on the non-absorbable phytate in the diet, thus reducing the chelation of calcium ions, and making more Ca^{2+} available for absorption.

The increase in the activity of alkaline phosphatase (EC 3.1.3.1) in response to vitamin D_3 has also been well documented.^{5,9,11} These results were suggestive of a role of alkaline phosphatase in Ca^{2+} transport process. The stimulation of alkaline phosphatase, however, occurs after the stimulation of Ca^{2+} transport.⁹ The physiological significance of this enzyme is therefore difficult to assess.

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The present paper reports, for the first time, the use of isolated rat small intestinal cells to study the distribution of the activities of Ca^{2+} -ATPase, phytase, and alkaline phosphatase along the length of the villus. The time course of changes in the levels of these enzymes in vitamin D-deficient rats on vitamin D_3 -repletion was followed.

Methods

MATERIALS

Vitamin D_3 ; ATP, disodium salt; p-nitrophenyl phosphate, disodium salt; phytic acid, sodium salt, and calf thymus DNA were purchased from Sigma Chemical Co, St Louis, Missouri. Ouabain was purchased from E Merck, Darmstadt, West Germany. Rachitogenic Diet no. 2, which contained 3.0% CaCO_3 , was a product of Nutritional Biochemicals, Cleveland, Ohio. Other chemicals were obtained from standard suppliers.

ANIMALS

Eight week old male rats and three week old male weanling rats of Sprague-Dawley strain were obtained from the University Laboratory Animal Unit. Vitamin D-deficient rats were raised by feeding the weanling rats with a vitamin D-free diet (Rachitogenic Diet no. 2) for five weeks. The rachitic animals had a body weight of 65–75 g as compared with normal eight week old rats, which weighed 220–250 g. To study the effect of vitamin D-repletion, the rachitic rats were given an intraperitoneal injection of 6.5 nmol (2.5 μg) vitamin D_3 in 0.05 cm^3 ethanol 12, 18, 24, 36, 48, 72, or 90 hours before the experiment. Control rats received ethanol only.

PREPARATION OF ISOLATED ENTEROCYTES

Villous and crypt cells were isolated by modifications of the method of Weiser.¹² In this method, citrate was used to solubilise the glycoprotein in the glycocalyx. This was followed by treatment with EDTA to remove calcium ions which are largely responsible for binding the cells together.

Rats were killed by cervical dislocation. A segment of the small intestine, 30 cm long, starting from the duodenal-jejunal junction was immediately removed and thoroughly rinsed with ice-cold 154 mM NaCl. The small intestine was filled with a solution (Solution A) composed of 1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4 , 5.6 mM Na_2HPO_4 , pH 7.3, and incubated at 37°C for 15 minutes. This first solution was discarded and the intestine was filled with a second solution (Solution B) composed of 10 mM Hepes, 150 mM sucrose, 40 mM KCl, 10 mM sodium succinate, 2

mM MgSO_4 , 25 mM NaCl, 1.5 mM EDTA, and 0.5 mM dithiothreitol, pH 7.3. This solution was chosen to obtain a completely dissociated, morphologically intact and viable cell suspension as shown by direct observation by light microscopy after staining with trypan blue. The intestine was then incubated at 37°C for increasing lengths of time of 4, 2, 2, 3, 4, 5, 7, 10, 15 minutes to sequentially detach the cells from villus tip to the crypts. The cells were readily released into the incubation medium by gently pressing the filled intestine with the finger. Nine sequential fractions of cells were collected.

The cells were pooled to give an upper villus fraction, a mid and lower villus fraction, and a crypt fraction. The pooled fractions were washed three times with the following ice-cold solution: 10 mM Hepes, 150 mM sucrose, 40 mM KCl, 10 mM sodium succinate, 2 mM MgSO_4 , 25 mM NaCl, pH 7.3, and finally suspended in the same solution at 4°C.

DETERMINATION OF ENZYME ACTIVITIES

All estimations were made at 37°C using 0.1 cm^3 cell suspension mixed with 1 cm^3 of the appropriate substrate. All reactions were linear over the time course chosen. In order to allow for direct comparisons of enzymatic activities the results were expressed as μmoles inorganic phosphate liberated per minute per μg DNA.

Calcium stimulated adenosine triphosphatase was measured by a modification of the method of Melancon and DeLuca.³ The reaction mixture consisted of 2.5 mM ATP, 10 mM CaCl_2 , 5 mM MgCl_2 , 154 mM NaCl, 50 mM Tris-HCl, pH 7.4. Calcium ions were omitted in the blank determinations. The incubation period chosen was 15 minutes.

Alkaline phosphatase was estimated by a modification of the method of Norman *et al.*¹³ Activity was measured after an incubation time of 15 minutes in a reaction mixture of 5 mM p-nitrophenyl phosphate, 0.2 mM ZnCl_2 , 0.5 mM MgCl_2 , 154 mM NaCl, 50 mM glycine, pH 9.5.

Phytase activity was measured after a 30 minute incubation using a reaction mixture composed of 2 mM phytic acid, 0.5 mM MgCl_2 , 154 mM NaCl, 50 mM Tris-HCl, pH 7.4.¹⁰

(Na^+ - K^+)-ATPase activity was measured according to Mircheff and Wright.¹⁴ The reaction mixture contained 3mM ATP, 100 mM NaCl, 10 mM KCl, 5 mM MgCl_2 , 3 mM Na_2EDTA , 100 mM Tris-HCl, pH 7.4. 0.5 mM ouabain was added in the blank determinations. Incubation was carried out for 15 minutes.

All reactions were stopped by the addition of 1 cm^3 ice-cold 10% (w/v) trichloroacetic acid. Tubes

were centrifuged at $1500 \times g$ for 15 minutes at 4°C . Inorganic phosphate was estimated in the supernatant by the method of Fiske and Subarow¹⁵ while the pellet was saved for DNA estimation.

DEOXYRIBONUCLEIC ACID ESTIMATION

The precipitated cell pellet was solubilised by warming with 1 cm^3 1% sodium lauryl sulphate at 60°C for 10 minutes.¹⁶ Sodium chloride was added to a final concentration of 0.5 M to the viscous, lysed suspension and the whole mixture was shaken with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) for 30 minutes. The resulting emulsion was centrifuged at $1500 \times g$ for 15 minutes. The upper aqueous layer was carefully removed for DNA estimation by the diphenylamine reaction.¹⁷ Calf thymus deoxyribonucleic acid was used as standard.

STATISTICAL METHODS

All results were expressed as a mean and standard error, and differences were compared by means of a Student's *t* test; differences of $p < 0.05$ were taken as significant.

Results

CELL PREPARATION

The method isolated enterocytes from different levels of the villus and crypt areas. The cells were generally well dispersed, but aggregates, which were particularly prominent in the crypt fraction were also present. The isolation process was monitored by staining the cells with methylene blue and observation by light microscopy. The first two to three fractions of cells isolated contained cells from the upper villus with typical columnar shape and a well defined brush border. In the following fractions, cells from the mid and lower villi were isolated. The cells were more rounded and shorter, brush border was also present. In the last fraction, only round crypt cells were present. They were relatively smaller and with lower cytoplasmic-nuclear ratio. In no case was there evidence of significant contamination with non-enterocytes, a finding confirmed by light microscopic examination of the intestine following the cell isolation procedure.

VILLUS-CRYPT GRADIENT OF ENZYMATIC ACTIVITIES

The distribution of Ca^{2+} -ATPase, phytase, and alkaline phosphatase activities along the villus of rat jejunum is shown in Fig. 1. A gradient of enzymatic activities existed along the villus and a similar distribution pattern was observed for the three

Fig. 1 Distribution of Ca^{2+} -ATPase, phytase, and alkaline phosphatase activities along the villus of normal and vitamin D-deficient rat jejunum.

Enterocytes were prepared and enzymes were assayed as described in Methods. Enzymatic activities are expressed as μmol of inorganic phosphate liberated per μg DNA. Each point represents mean value of 10 to 17 experiments $\pm$ SEM.

● Enzymatic activities in normal rats. ○ Enzymatic activities in vitamin D-deficient rats. U-V represents upper villus fraction, M-V mid-villus fraction, L-V lower villus fraction, and C crypt fraction. *** $p < 0.001$ compared with normal; ** $p < 0.01$ compared with normal controls.

enzymes studied. In all cases, the enzymatic activities were the highest in the upper villus fraction, with a gradual decrease to low level in the crypts. The activities were eight- to ten-fold greater in cells of the villus tip than those of the crypt base.

The $(\text{Na}^+ - \text{K}^+)$ -stimulated, ouabain-sensitive ATPase activity was also followed. As shown in Table 1, $(\text{Na}^+ - \text{K}^+)$ -ATPase displayed an even

Table 1 Distribution of (Na^+ - K^+)-stimulated and ouabain-sensitive ATPase along villus of rat jejunum

	ATPase activity		
	Total	+ Ouabain	(Na^+ - K^+)-ATPase
Upper villus	0.619±0.028	0.509±0.046	0.110±0.020
Mid-villus	0.484±0.025	0.366±0.037	0.118±0.013
Lower villus	0.304±0.037	0.191±0.022	0.113±0.022
Crypts	0.166±0.027	0.110±0.017	0.056±0.014

Enzymatic activities are expressed as μmoles of inorganic phosphate liberated per minute per $100 \mu\text{g}$ DNA. Mean values $\pm$ SEM for four experiments are given. (Na^+ - K^+)-ATPase activity is taken as the difference between total ATPase activity and ATPase activity in the presence of 0.5 mM ouabain.

distribution throughout the villus. An attempt was made to differentiate between Ca^{2+} -stimulated and (Na^+ - K^+)-stimulated ATPase activities. Ouabain, which inhibited (Na^+ - K^+)-ATPase, had no effect on the Ca^{2+} -stimulated ATPase.

KINETIC STUDIES

The activities of the enzymes in the cells from the villus tip and crypt were determined at various substrate concentrations. Table 2 shows that the K_m values between the two cell fractions in particular that of Ca^{2+} -ATPase, are very different with the lowest values being found in the villus cells. The K_m of phytase in the crypt fraction was not determined owing to low enzymatic activity and insensitivity of the assay method.

EFFECTS OF VITAMIN D REPLETION

To determine whether a differential response in the activity levels of Ca^{2+} -ATPase, phytase, and alkaline phosphatase to vitamin D_3 occurs along the villus, 6.5 nmoles ($2.5 \mu\text{g}$) of vitamin D_3 was injected intraperitoneally into rachitic rats and changes in enzymatic levels of the enterocytes isolated from the villus and the crypts were followed at 12, 18, 24, 36, 48, 72, and 90 hours after vitamin D-repletion. The results are shown in Fig. 2.

Recovery of the activities of all the three enzymes was observed in all areas of the villus after vitamin D_3 repletion, but the rates of recovery were markedly different. Vitamin D_3 treatment did not alter the activities of these enzymes in the crypt cells.

There was no significant rise in the enzymatic levels of Ca^{2+} -ATPase 12 hours after administration of vitamin D_3 , but the activity reached normal levels by 18 hours. As shown in Fig. 2, all the three villus cell fractions responded in roughly parallel fashion. The enzymatic level was further increased up to 72

hours, after which it gradually declined.

Increase in phytase activity was observed in rachitic rats after vitamin D_3 administration. Total recovery of activity to normal levels was observed by 24 hours. A further rise in enzymatic activity was apparent.

The level of alkaline phosphatase gradually increased 18 hours after vitamin D_3 treatment. By 36 hours, the activity in all areas of the villus had risen to the normal level, and remained so throughout the rest of the experiment.

Discussion

The small intestinal mucosa consists of mitotically active crypt cells which migrate gradually towards the villus tip, differentiating from secretory to absorptive cells. Until recently¹² it has not been possible to study this differentiation process in isolated enterocytes free from contamination with cells from the lamina propria and the muscle layers.

The present study has described the distribution of vitamin D-dependent enzymes in enterocytes from normal, vitamin D-deficient and -replete rats. Previous studies, using various techniques¹²⁻¹⁸⁻²⁰ have shown that alkaline phosphatase is absent, or at very low levels, in the crypts with a gradual increase to a maximum at the villus tip. We have found that a similar pattern of distribution is shown by both Ca^{2+} -ATPase and phytase. The activity of (Na^+ - K^+)-ATPase, a marker for baso-lateral membranes¹⁴ was constant in all villous fractions, activity only being reduced in the crypts.

The relationship between Ca^{2+} -ATPase, phytase and alkaline phosphatase, and their role in calcium transport is not well understood. A continuing

Table 2 Comparison of Michaelis constants of Ca^{2+} -ATPase, phytase, and alkaline phosphatase between villus tip and crypt

	K_m (mM)	
	Upper villus	Crypt
Ca^{2+} -ATPase	0.28±0.03 ^a (7)	0.90±0.05 ^d (4)
Phytase	0.17±0.01 ^b (14)	ND
Alkaline phosphatase	0.25±0.03 ^c (8)	0.67±0.10 ^e (4)

Epithelial cells from normal rat jejunum were isolated as described in Methods. The upper villus fraction and the crypt fraction were used for kinetic experiments. K_m values were calculated from Lineweaver-Burk plots of enzymatic activity against substrate concentration.

Results are given as the mean value $\pm$ SEM. Figures in parentheses represent the number of experiments performed.

a vs b = $p < 0.001$; a vs c = difference not significant;

a vs d = $p < 0.001$; d vs e = $p < 0.02$; c vs e = $p < 0.001$.

ND = not detectable owing to very low enzymatic activity.

Fig. 2 Time course of recovery of Ca^{2+} -ATPase, phytase, and alkaline phosphatase activities along the villus or rachitic rat jejunum in response to vitamin D_3 treatment.

Rachitic rats received an intraperitoneal dose of $2.5 \mu\text{g}$ (6.5 nmol) vitamin D_3 , and were killed at appropriate times. Enzymatic activities were assayed as described in Methods. Activity in normal rat is taken as 100% and recovery is the difference between that of D_3 -repleted animal and rachitic control. Results are expressed as percentage recovery as compared with the activities found in normal rats. Each point represents the mean of 4 to 6 experiments $\pm$ SEM.

●—● Ca^{2+} -ATPase; ▲—▲ phytase; ■—■ alkaline phosphatase.

controversy involves the possibility that Ca^{2+} -ATPase and phytase are identical with or isoenzymes of the brush border alkaline phosphatase.^{8 10 11} On vitamin D-depletion the activities of all three enzymes were reduced in the villous cells, but not in the crypt cells, suggesting identity between the enzymes. Experiments on the kinetic properties and time course of recovery after vitamin D-repletion, however, lead to a different conclusion.

The Kms of alkaline phosphatase and phytase obtained, with the upper villus fraction are in good agreement with previously reported values.²¹ There were, however, very marked differences between the Kms of both alkaline phosphatase and Ca^{2+} -ATPase between the villus tip and the crypt cells. These data show that, during differentiation, not only does the apparent enzymatic activity increase but also the affinity of the enzymes for their substrate. It is not possible to conclude that the enzymes are different entities or whether some conformational change occurs during differentiation.

Most of the previous studies on the effects of vitamin D_3 - or $1,25\text{-(OH)}_2\text{D}_3$ -repletion aimed only at comparing the enzymatic activities of the animal before and after repletion. Comparison of the enzymatic activities of rachitic rats upon vitamin D-repletion with those in normal controls have not

been attempted. Furthermore, in most previous work, homogenates of mucosal scrapings or frozen sections were used. Our data show that different recovery rates were exhibited by the three enzymes in response to vitamin D treatment. In all the three villus cell fractions the fastest response was that of Ca^{2+} -ATPase while alkaline phosphatase was the slowest. With the exception of alkaline phosphatase, further stimulation was observed after the enzymatic activities had increased to the level of normal control rats. Maximal development of the physiological response to a dose of 6.5 nmol ($2.5 \mu\text{g}$) vitamin D_3 requires approximately 70 hours, at which time the activities of Ca^{2+} -ATPase and phytase become almost doubled that of the normal rats. After vitamin D_3 is given to the rachitic rats, there is a time lag of about 12 hours before significant rise in enzymatic activities can be detected. Part of this lag period can be attributed to the time required for the conversion of vitamin D_3 to its active form, $1,25\text{-(OH)}_2\text{D}_3$.²² The continuous rise in enzymatic activities of Ca^{2+} -ATPase and phytase may be because of surplus production of $1,25\text{-(OH)}_2\text{D}_3$ in the D-repleted animal which further exerts its affect on the mucosa. The gradual fall in enzymatic activities after the maximal values had been reached may be due to gradual fall in tissue level of vitamin D_3 and thus $1,25\text{-(OH)}_2\text{D}_3$, coupled with the turnover of the intestinal mucosa.

The fast response of Ca^{2+} -ATPase to vitamin D_3 -repletion and the upsurge of its activity to a very high value by 60 hours reflect the possibility that this enzyme is directly involved in Ca^{2+} absorption. These effects may lead to increased efficiency of absorption of Ca^{2+} by the villi in order to raise the blood Ca^{2+} concentrations back to normal. The observed response of phytase supports the view that this enzyme has an indirect role in calcium absorption.¹⁰ The gradual drop in activity levels of Ca^{2+} -ATPase and phytase may also be due to the feedback effect as a result of the rise of plasma Ca^{2+} and a fall in parathyroid hormone secretion which stops further conversion of vitamin D_3 to $1,25\text{-(OH)}_2\text{D}_3$. Different response was exhibited by alkaline phosphatase, suggesting that it may not have a definite role in Ca^{2+} absorption. Current reports indicated that the increase in Ca^{2+} -ATPase in response to $1,25\text{-(OH)}_2\text{D}_3$ is in parallel with the increased calcium absorption.⁹ These observations support our findings well.

The vitamin D status of the animal did not influence the activities of Ca^{2+} -ATPase, phytase, or alkaline phosphatase in the crypt cells. No difference in enzymatic activities was observed between crypt cells isolated from normal and rachitic rats. Furthermore there was no change in activities after administration of vitamin D_3 to rachitic rats. This suggests that these enzymes in the crypt cells may not be vitamin D-dependent. The difference in kinetic properties between crypt and villus tip cells, and the vitamin D-independent nature of the crypt cells suggest that the crypt cells may not possess $1,25\text{-(OH)}_2\text{D}_3$ receptors and the latter only appear after the cells have migrated towards the villus and differentiated into absorptive cells.

In summary, our study shows that the three vitamin D-dependent enzymes, namely, Ca^{2+} -ATPase, phytase, and alkaline phosphatase, are of different identity, and that they are due to three distinct enzymes or different manifestation of an enzyme complex. Our results are also suggestive that the activities of these enzymes in the crypt cells may be independent of the vitamin D status of the animal.

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