THE EFFECT OF 1,25-DIHYDROXYCHOLECALCIFEROL ON THE MULTIPLE FORMS OF ALKALINE PHOSPHATASE AND THE SIALIC ACID INCORPORATION INTO MICROSONES OF CHICK DUODENUM

Sachiko MORIUCHI, Setsuko YOSHIZAWA and Norimasa HOSOYA

Department of Nutrition, School of Health Sciences, Faculty of Medicine, University of Tokyo, 3-1, Hongo 7, Bunkyo-ku, Tokyo 113, Japan
(Received May 24, 1977)

Summary Polyacrylamide disc gel electrophoresis of n-butanol solubilized alkaline phosphatase from chick duodenum revealed that the change of alkaline phosphatase induced by 1,25-(OH)₂D₃ involved the transformation of desialoenzyme to sialoenzyme. The initial stimulation by 1,25-(OH)₂D₃ of the incorporation of sialic acid into duodenal microsomes corresponded with the initial increase in calcium absorption. After this initial stimulation, there was a rapid decline in sialic acid incorporation into microsomes decreasing below control levels at 24 hr. Calcium concentration in the microsomes followed a pattern similar to the incorporation of sialic acid into microsomes. The depressed sialic acid incorporation was reversed by the addition of calcium in vitro. These results suggest that the initial action of 1,25-(OH)₂D₃ is to change the membrane permeability to calcium and to change the subcellular distribution of calcium in the small intestine. The accumulated calcium in the microsomes then stimulates the sialic acid incorporation into desialoenzyme. This results in the changes of isozyme pattern of alkaline phosphatase, viz, the transformation of desialoenzyme to sialoenzyme. The transformed alkaline phosphatase might be one of the factors involved more directly in the regulation of calcium transport in intestine.

Recently, MORIUCHI and DELUCA demonstrated that a chick intestinal brush border protein was modified by 1,25-dihydroxycholecalciferol (1,25-(OH)₂D₃) prior to the appearance of calcium binding protein (CaBP) and this modification was closely correlated with the initial increase in intestinal calcium absorption. The protein which undergoes the change possesses alkaline phosphatase (ortho-
phosphoric monoester phosphohydrolase [EC 3.1.3.1]) activity (1).

The n-butanol solubilized alkaline phosphatase from chick duodenum has been resolved into three groups of molecules. On electrophoresis the two isozymes show fast moving bands differing in their association with sialic acid (2).

In order to examine whether the change of brush border protein induced by 1,25-(OH)2D3 can be ascribed to the changes in isozyme pattern of alkaline phosphatase, the protein was solubilized by n-butanol and subjected to electrophoresis. The results are discussed in relation to the sialyltransferase activity and calcium concentration of intestinal microsomes.

METHODS

Animals. One-day-old White Leghorn chicks were raised on rachitogenic diet (3) for 4–5 weeks to obtain vitamin D-deficient chicks. Vitamin-D-deficient chicks were dosed with either 325 pmoles of 1,25-(OH)2D3 in 50 μl of 95% ethanol or 50 μl of 95% ethanol intravenously via the wing vein at various times before sacrifice.

Tissue preparation. Duodenal mucosa of vitamin D-deficient chicks dosed with 125 ng of 1,25-(OH)2D3 24 hr before sacrifice and those without it were frozen and used for the extraction of alkaline phosphatase by n-butanol. Microsomes of duodenal mucosa were prepared from 1,25-(OH)2D3 dosed and undosed vitamin-D-deficient chicks sacrificed 1, 3, 6 and 24 hr after the dose was given.

Preparation of enzyme extract and digestion with neuraminidase. For the study of alkaline phosphatase isozymes in chick duodenum, alkaline phosphatase was solubilized from chick duodenal mucosa by n-butanol as described in the previous report (1). An n-butanol extract (50 μl containing 45-60 μg protein) was incubated with 10 μl of 0.01 M MgCl2, 10 μl of 0.01 M CaCl2 and 50 μl of 0.4 M sodium acetate buffer at pH 5.3 in the presence or absence of 100 μl neuraminidase (Vibrio Cholerae, 50 units activity, Calbiochem.). After incubation at 37°C for 1 hr, the digests were subjected to electrophoresis without further treatment.

Electrophoresis. An aliquot of n-butanol extract, with or without neuraminidase treatment, was subjected to electrophoresis using essentially the method of Davis (4). Acrylamide concentration of the running gel was 5.3%. After electrophoresis, the gels were incubated in a solution containing 0.033 M Tris-HCl, pH 9.5; 0.025 M sodium α-naphthylphosphate and 1 mg/ml of Fast Red TR (diazonium salt of 4-chloro-o-toluidine) at room temperature. After the bands appeared, the gels were removed from the substrate solution and washed several times with distilled water (5).

Preparation of duodenal microsomes. Crude chick duodenal microsomes were prepared from 10% (w/v) homogenate in 0.25 M sucrose. The homogenate was centrifuged at 10,000 × g for 10 min and the microsomes sedimented at 105,000 × g for 60 min. The microsomes were suspended in 0.25 M sucrose and used for
determination of calcium. For the study of sialic acid incorporation, the microsomes were desialylated according to the method of Järnefelt (6). The microsome suspension containing about 20 mg of protein was incubated for 1 hr with 50 units of Vibrio Cholerae neuraminidase in the presence of 2 mM CaCl$_2$ in 20 mM sodium acetate buffer, pH 5.6 in a final volume of 2 ml. After the addition of 5 $\mu$mol of EDTA (ethylene diamine tetraacetic acid) and 50 $\mu$mol MES buffer (2(N-morholino) ethanesulfonic acid), pH 6.3, the mixture was diluted with 10 ml of 0.25 M sucrose and centrifuged for 60 min at 105,000 × g. The precipitates were resuspended in 0.25 M sucrose and used for sialic acid incorporation (desialylated microsomes).

Incorporation of sialic acid. Incorporation of sialic acid into desialylated microsomes was measured in an incubation mixture of the following composition (6): 20 mM MES buffer, pH 6.3, containing 0.25% Triton X-100, 0.05 $\mu$Ci CMP-N-acetyl-[4,5,6,7,8,9-$^{14}$C]neuraminic acid in ammonium bicarbonate buffer (specific activity 200 Ci/mol, New England Nuclear). Total volume was 100 $\mu$l. After incubation for 60 min, the reaction was stopped with 1 ml of 12% cold trichloroacetic acid, and kept at least 10 min in an iced bath. The precipitate was separated on Whatman GF/A fiber-glass filters, washed with 3 ml of 12% trichloroacetic acid, then rinsed with 10 ml cold acetone to remove trichloroacetic acid on the filter. Filter discs were dried for 20 min at 100°C. The radioactivity was counted by liquid scintillation in 10 ml of toluene scintillator containing 4% Omniflour (New England Nuclear) using a Packard Tricarb liquid scintillation spectrometer model 3380. The counts are given as cpm/mg protein.

Assay procedures. Alkaline phosphatase was assayed with p-nitrophenylphosphate as a substrate in 2-amino-2-methyl-1-propanol at pH 10 (7). Activity is expressed as $\mu$mol of p-nitrophenol released per min per mg protein. Protein was determined by the method of Lowry et al. (8). To prepare the samples for calcium assay, duodenal microsomes were dried in an oven at 110°C for 1 hr, then ashed in a muffle furnace at 600°C overnight. The ashed microsomes were solubilized in 2 ml of 2 N HCl and calcium was determined by atomic absorption spectroscopy.

Compounds. 1,25-(OH)$_2$D$_3$ was kindly provided by Dr. H. F. DeLuca (Department of Biochemistry, University of Wisconsin, USA). CMP-N-acetyl-[4,5,6,7,8,9-$^{14}$C]neuraminic acid was obtained from New England Nuclear. Neuraminidase (Vibrio Cholerae) was from Calbiochem. Other reagents were conventional analytical grade chemicals.

RESULTS

1. The effect of 1,25-dihydroxycholecalciferol on the multiple forms of alkaline phosphatase in chick duodenum

Morichii and DeLuca showed that an intestinal brush border protein is
modified prior to the appearance of CaBP by 1,25-(OH)₂D₃ and this modification was observed at 6 hr after 1,25-(OH)₂D₃ administration and returned to that of the vitamin D-deficient chicks after 48 hr; this is in good correlation with the increase in calcium absorption (I). To examine whether the change in the duodenal brush border protein of vitamin D-deficient chick induced by 1,25-(OH)₂D₃ is a result of the change in the multiple forms of alkaline phosphatase, the protein was solubilized by n-butanol from vitamin D-deficient chick duodena and those dosed with 1,25-(OH)₂D₃ 24 hr before sacrifice and subjected to electrophoresis.

Polyacrylamide disc gel electrophoresis revealed that the change in alkaline phosphatase induced by 1,25-(OH)₂D₃ involved the transformation of desialo-enzyme to sialoenzyme (Fig. 1). Four alkaline phosphatase bands were observed:

![Fig. 1. Electrophorograms of n-butanol solubilized alkaline phosphatase of chick duodenum.](image)

Electrophoresis in 5.3% polyacrylamide gels was toward the anode (bottom). 1, N-Butanol extract of duodenal mucosa homogenate from rachitic chicks (without neuraminidase treatment); 2, same as 1 except for that the extract was treated by neuraminidase; 3, same as 1 except for that the extract was from 1,25-(OH)₂D₃-treated rachitic chicks; 4, neuraminidase treated extract from 1,25-(OH)₂D₃-treated chicks. Alkaline phosphatase bands were designated as A, B, C, and D according to the mobility.

three near the origin and one migrating further, which were designated as bands A, B, C and D. Band C and band D of vitamin D-deficient chick duodenum migrated further in 1,25-(OH)₂D₃ repleted chick duodenum. However, the mobility of bands C and D of chick duodenum was decreased by neuraminidase treatment prior to electrophoresis (Fig. 1). This suggests that bands C and D contain sialic acid.
2. The effect of 1,25-dihydroxycholecalciferol on the incorporation of sialic acid into chick duodenal microsomes

The results of electrophoresis of the n-butanol extract suggested that 1,25-(OH)\textsubscript{2}D\textsubscript{3} might be involved in the process of sialyzation of the desialoenzyme. Therefore, sialyltransferase activity was determined in the microsomal fraction of vitamin D-deficient and 1,25-(OH)\textsubscript{2}D\textsubscript{3} dosed chick duodenal mucosa. Three hours after the dose, the activity of this enzyme was significantly stimulated. However, after this initial stimulation, there was a rapid decline in sialic acid incorporation into microsomes, decreasing below control levels at 24 hr; it was reduced to less than half of that found in vitamin D-deficient animals (Fig. 2). It has been reported that calcium transport is still high and CaBP appears in the duodenal mucosa at this time (1). The addition of calcium to the incubation medium resulted in a stimulation of sialic acid incorporation into duodenal microsomes from vitamin D-deficient chicks dosed with 1,25-(OH)\textsubscript{2}D\textsubscript{3} 24 hr before sacrifice (Table 1).

3. The effect of 1,25-dihydroxycholecalciferol on the calcium content of duodenal microsomes

The reversal of the depressed sialic acid incorporation by the addition of calcium in vitro suggested that the level of calcium of the duodenal microsomes might regulate sialic acid incorporation into protein. Therefore, it was important to study the effect of 1,25-(OH)\textsubscript{2}D\textsubscript{3} on the calcium content of duodenal microsomes.
Table 1. Effect of calcium on sialic acid incorporation into duodenal microsomes from vitamin D-deficient chicks dosed with 1,25-(OH)₂D₃ 24 hr before sacrifice.

| Addition (CaCl₂) | Sialyltransferase activity (cpm/mg protein) |
|------------------|------------------------------------------|
| None             | 1.50                                     |
| 1 x 10⁻⁴ M       | 3.60                                     |
| 1 x 10⁻⁵ M       | 3.45                                     |

Sialyltransferase activity was assayed in the duodenal microsomes from vitamin D-deficient chicks dosed with 1,25-(OH)₂D₃ in the presence or absence of Ca²⁺. 125 ng of 1,25-(OH)₂D₃ was dosed to vitamin D-deficient chicks 24 hr before sacrifice.

The changes in calcium concentration of microsomes followed a pattern similar to the incorporation of sialic acid into protein (Fig. 2). Three hours after dosing with 1,25-(OH)₂D₃, the calcium content of duodenal microsomes was increased, thereafter, it decreased rapidly and at 24 hr after the dose had fallen to below control levels.

DISCUSSIONS

The present study demonstrates that the change in chick duodenal brush border protein, induced by 1,25-(OH)₂D₃, involves the sialization of alkaline phosphatase variants.

Chang and Moog showed that chick duodenal alkaline phosphatase can be resolved into several forms (F₁, F₂, and S) by electrophoresis on starch gels. Two forms, F₁ and F₂, migrated fast in this system. F₁ was shown to be a sialoenzyme and migrated slightly further than F₂, while the S-form moved slowly. In our studies, polyacrylamide disc gel electrophoresis of n-butanol extracts gave a migration pattern similar to that described for the starch gels. Our findings using the neuraminidase treatment of n-butanol extracts suggest that band A and band B of vitamin D-deficient and 1,25-(OH)₂D₃ treated chicks correspond to the S-enzyme described above, while band D of the vitamin D-deficient chicks corresponds to F₂ and the band from 1,25-(OH)₂D₃ treated chicks to F₁.

We found that the calcium content of microsomes increased three hours after the administration of 1,25-(OH)₂D₃ and that this correlated with the increase in sialyltransferase activity. The stimulation of sialyltransferase activity of duodenal microsomes occurred prior to the changes in chick duodenal brush border protein and the activation of calcium transport in response to 1,25-(OH)₂D₃ (I), and was considerably reduced after 24 hr, at the time when the induction of CaBP occurs (I). Thus, although there have been numerous suggestions that CaBP may be directly involved in calcium transport in the intestine (9-10), this interpretation is subject to criticism, because the appearance of CaBP is not closely correlated with the stimulation of calcium transport by 1,25-(OH)₂D₃ (I, 11-14).

The above observations suggest that 1,25-(OH)₂D₃ initially alters membrane
permeability to calcium ion, which then causes a change in the subcellular distribution of this ion. This concept is also supported by our studies with vitamin D₃ on the rat intestine where we observed the changes in subcellular distribution of calcium prior to the appearance of CaBP (15, 16). Another illustration of vitamin D₃ effect on membranes is the reported calcium release from mitochondria by the stimulation with vitamin D₃ in vivo and in vitro (17, 18).

We would like to propose that the initial action of 1,25-(OH)₂D₃ is to alter the subcellular distribution of calcium in the small intestine through a change in the membrane permeability of calcium. An increased calcium level in the microsomes then stimulates the incorporation of sialic acid into alkaline phosphatase. This transformed alkaline phosphatase is one of the factors responsible for the more direct regulation of intestinal calcium transport. On the other hand, nuclear 1,25-(OH)₂D₃, which was transported to nuclei by cytosol receptor mechanism (19, 20), as the mechanism elucidated for steroid hormone (21), may induce CaBP, although its appearance is after the stimulation of calcium transport (1, 11–14). The function of the induced CaBP is not clear, but it could be to sequester calcium from microsomes so as to regulate or terminate the action of 1,25-(OH)₂D₃.

We would like to thank Dr. H. F. DeLuca (University of Wisconsin, USA) for his suggestions on this study and for providing us with 1,25-(OH)₂D₃. We also would like to thank Dr. M. Zile for her critical reading of our manuscript.

REFERENCES

1) MORIUCHI, S., and DELUCA, H. F. (1976): The effect of vitamin D₃ metabolites on membrane proteins of chick duodenal brush borders. Arch. Biochem. Biophys., 174, 367–372.
2) CHANG, C-H., and MOOG, F. (1972): Alkaline phosphatase of chicken duodenum. I. Isolation and partial characterization of the multiple forms of duodenal phosphatase in pre- and post-hatching stages. Biochim. Biophys. Acta, 258, 154–165.
3) OMDAHL, J., HOLICK, M., SUDA, T., TANAKA, Y., and DELUCA, H. F. (1971): Biological activity of 1,25-dihydroxycholecalciferol. Biochemistry, 10, 2935–2940.
4) DAVIS, B. J. (1964): Disc Electrophoresis-II. Method and application to human serum proteins. Ann. New York Acad. Sci., 121, Art. 2, 404–427.
5) ALLEN, J. M., and HYNCIK, G. (1963): Localization of alkaline phosphatase in gel matrices following electrophoresis. J. Histochem., 11, 169–175.
6) JÄRNEFELT, J. (1976): Protein-lipid interactions in the sialic acid incorporating system of liver microsomes. Biochim. Biophys. Acta, 428, 711–719.
7) LOWRY, O. H. (1957): Micromethods for the assay of enzymes. II. Specific procedure, alkaline phosphatase. in Methods in Enzymology, Vol. 4, ed. by Colowick, S. P., and Kaplan, N. P., Academic Press, New York, pp. 371–372.
8) LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265–275.
9) TAYLOR, A. N., and WASSERMAN, R. H. (1969): Correlations between the vitamin D-induced calcium binding proteins and intestinal absorption of calcium. Fed. Proc., 28, 1834–1838.
10) CORRADINO, R. A., and WASSERMAN, R. H. (1973): Vitamin D, calcium, and protein
synthesis. *Vitam. and Horm.*, 71, 41–67.
11) SPENCER, R., CHARMA, M., WILSON, P., and LAWSON, D. E. M. (1976): Vitamin D-stimulated intestinal calcium absorption may not involve calcium-binding protein directly. *Nature*, 263, 161–163.
12) LAWSON, D. E. M. (1977): Recent studies on 1,25-(OH)2D3 action in the intestine. *in* Third Workshop on Vitamin D, Abstract p. 64, Asilomar Conference Grounds, Pacific Grove, California, Jan. 9–13.
13) RASMUSSEN, H., MAX, E., and GOODMAN, D. B. P. (1977): Effect of 1,25-(OH)2D3 on the composition of the chick intestinal brush border membrane. *ibid.*, p. 69.
14) BIKE, D. D., ZOLLOCK, D. T., MORRISSEY, R. L., and HERMAN, R. H. (1977): 1,25-(OH)2D3-induced CaBP production and alkaline phosphatase activity from calcium transport by actinomycin D and cycloheximide. *ibid.*, p. 56.
15) ITTOYO-SUGISAKI, N., MORIUCHI, S., and HOSOYA, N. (1975): Vitamin D3 effect on the calcium content in the subcellular fractions of rat intestinal mucosa. *J. Nutr. Sci. Vitaminol.*, 21, 235–236.
16) MORIUCHI, S., YAMANOUCHI, T., and HOSOYA, N. (1975): Demonstration of two different vitamin D-dependent calcium binding proteins in rat intestinal mucosa., *J. Nutr. Sci. Vitaminol.*, 21, 251–259.
17) ENGSTROM, G. W., and DELUCA, H. F. (1962): The action of vitamin D in vivo and in vitro on the release of calcium from kidney mitochondria. *J. Biol. Chem.*, 237, PC974–PC975.
18) ENGSTROM, G. W., and DELUCA, H. F. (1964): Vitamin D-stimulated release of calcium from mitochondria. *Biochemistry*, 3, 203–209.
19) LAWSON, D. E. M., and WILSON, P. W. (1974): Intranuclear localization and receptor proteins for 1,25-dihydroxycholecalciferol in chick intestine. *Biochem. J.*, 144, 573–583.
20) BRUMBAUGH, P. F., and HAUSLER, M. R. (1974): 1α,25-Dihydroxycholecalciferol receptors in intestine. II. Temperature-dependent transfer of the hormone to chromatin via specific cytosol receptor. *J. Biol. Chem.*, 249, 1258–1262.
21) YAMAMOTO, K. R., and ALBERTS, B. M. (1976): Steroid receptors: Elements for modulation of eucaryotic transcription. *Ann. Rev. Biochem.*, 45, 721–746.