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EVIDENCE FOR A 1,25-DIHYDROXYCHOLECALCIFEROL-DEPENDENT SPERMINE-BINDING PROTEIN IN CHICK DUODENAL MUCOSA

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SUMMARY

The spermine-binding activity of a cytosol protein fraction from chick duodenal mucosa changes in relation to the circulating level of 1,25-dihydroxycholecalciferol. The spermine-binding activity increases very rapidly within 1-2 hours after the rachitic chick was dosed intracardially with 1,25-dihydroxycholecalciferol. The clear and reproducible response is prevented by actinomycin D and cycloheximide. This increase is one of the earliest events induced by the active form of vitamin D_3 in the duodenal cell of rachitic chicks.

INTRODUCTION

We recently reported (1, 2) the existence of a selective spermine-binding protein in the cytosol fraction of chick duodenal mucosa. This protein is heat-sensitive, has a molecular weight of about 32,000 D, and from competition studies (3), it appears that this protein is not the well known duodenal calcium-binding protein (4).

The spermine-binding protein is already present in the duodenal cell of chick embryo and its activity is remarkably increased after hatching and even more after 7 days of life (2).

We report here that the administration of 1,25-$(OH)_2D_3$* to rachitic chicks rapidly stimulate the spermine-binding activity of the duodenal protein fraction. New protein synthesis appears to be necessary for this action of vitamin D.

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*Abbreviation: 1,25-$(OH)_2D_3$ = 1,25-dihydroxycholecalciferol.

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MATERIALS AND METHODS

Chemicals. [^{3}H] Spermine tetrahydrochloride (44.3 Ci/mmole) was obtained from New England Nuclear. Electrophoretically pure nucleases purchased from Worthington, were always desalted before use. Cycloheximide, Actinomycin D and cold spermine were obtained from Sigma. 1,25-Dihydroxycholecalciferol was generously provided by Prodotti Roche, Milano (Italy). Animals used in all experiments were White Leghorn cockerels that were raised for 3-4 weeks on a vitamin D-deficient diet (5). Experiments were carried out in duplicate and/or were repeated at least four times to assure reliability. Vitamin D dosed chicks received 140 ng of 1,25-$(OH)_2D_3$ intracardially in 0.1 ml of ethanol/propylene glycol (1:9 v/v) before being killed at the times stated. The control animals received injections of the same amount of vehicle. All chicks were starved for 16 hrs before death.

All manipulations were carried out at 0-2°. The duodenum was removed, rinsed in 40 mM Tris-HCl, pH 7.5, and the mucosal layer separated from the underlying muscle layers using a glass slide. The mucosa was homogenized in two volumes of the same buffer with a Potter-Elvehjem homogenizer and teflon pestle. The homogenate was centrifuged at 105,000 x g for 1 hr. The upper three quarters of supernatant were taken and used as the cytosol fraction.

The cytosol fraction (150-200 mg of protein) was then chromatographed on a DEAE-cellulose (Whatman DE-52) column (1 x 25 cm) that was equilibrated with the same buffer. The column was eluted stepwise, with buffer containing 0.1, 0.2, and 0.3 M KCl. The protein fractions were dialyzed extensively against 40 mM glycine buffer, pH 8.7, and assayed for their spermine-binding activity.

The spermine-binding activity of the freshly fractionated duodenal proteins was analyzed by gel filtration on Sephadex G-25 as described previously (1). Since RNAse treatment of the protein sample does not affect spermine-binding activity (1) in all the experiments the sample was digested with a protease-free solution of RNAse (pancreatic A + T_1, 20:1) to minimize the error of measurement due to RNA molecules, eventually contaminating the preparation. The protein sample (100-300 µg) was incubated with 0.2 µCi of 15 µM [^{3}H] spermine in 0.35 ml of 40 mM glycine buffer, pH 8.7, at 0°C for 10 min. After incubation the reaction mixture was passed through a Sephadex G-25 column (0.7 x 16 cm) and 0.5 ml fractions collected for the measurement of radioactive spermine bound to the protein in the void volume of the effluent (1).

Protein concentration was measured by the method of Warburg and Christian (6).

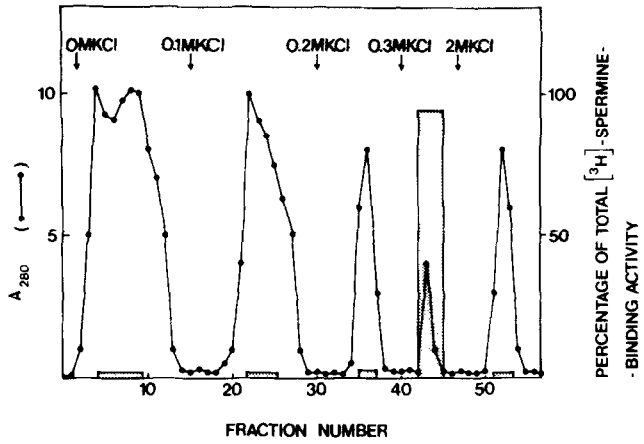

Fig. 1: Preparative DEAE-cellulose column chromatography of cytosolic spermine-binding protein. 400 mg of cytosol protein were applied to a DEAE-cellulose column (1.5 x 13 cm) that was equilibrated with 40 mM Tris-HCl, pH 7.5. The column was then eluted stepwise with the same medium containing increasing concentrations of KCl as shown in figure. The effluent was collected in 3-5 ml fraction starting to collect at the beginning of peak eluted by the equilibrating buffer. All the fractions peaks were pooled separately and dialyzed extensively against 40 mM glycine pH 8.7. Aliquots from each fraction (0.140 OD_{280}) were digested with 30 µg of a RNAse mixture (pancreatic + T_1 20:1) for 20 min at 25°C and then incubated with the radioactive spermine as described under the Methods section. Vertical bars represent the distribution of the total cytoplasmic binding activity into the different elution peaks.

RESULTS AND DISCUSSION

Fig. 1 shows the DEAE-cellulose column chromatography pattern of duodenal mucosa cytosol from rachitic chicks injected with 1,25-$(OH)_2D_3$. More than 90% of the cytosol protein is eluted from the column by the medium containing 0.2 M KCl, but these proteins do not have significant spermine-binding activity. Almost all the spermine-binding protein is eluted from the column by the medium with 0.3 M KCl, whereas the major contaminating RNA is eluted at KCl concentrations higher than 0.3 M.

The cytosol fraction from duodenal mucosa of vitamin D deficient chick gives a qualitatively similar chromatographyc pattern but the protein fraction eluted from DEAE-cellulose with 0.3 M KCl shows much lower spermine-binding activity.

In some experiments, after DEAE-cellulose chromatography the active fraction was passed through a column of Concanavalin A-Sepharose. The spermine-binding activity was almost totally recovered in the unbound fraction.

The effect of 1,25-$(OH)_2D_3$ can also detected using crude cytosol preparations, however, it is preferable to use the protein fractions previously purified by DEAE-cellulose chromatography. For this reason we studied the effect of 1,25-$(OH)_2D_3$ by comparing the spermine-binding activities of the protein fractions which are retained by a DEAE-cellulose column and which can be eluted from the column by 0.3 M KCl but not by 0.2 M KCl. All the protein preparations assayed in these experiments were freshly made and run in parallel with the proper control.

Fig. 2 reports the time-course response of duodenal spermine-binding activity to 140 ng of 1,25-$(OH)_2D_3$ injected intracardially. The increase is negligible within the first 30 min after dosing; this lag phase is followed by a period up to 2.5 hrs of rapid and remarkable increase in the activity. Thereafter the activity increased slower and eventually reached a plateau value.

The time course effect of 1,25-$(OH)_2D_3$ on the spermine-binding activity is very rapid and might involve either synthesis of new molecules of binding protein and/or an activation of preexistent molecules of a less active form. In Fig. 3 is shown that the stimulating effect of 1,25-$(OH)_2D_3$ was almost totally abolished when a large dose of cycloheximide was administered to the vitamin D-deficient chicks 1.5 hr prior to the hormone injection.

In Fig. 4 is reported that the activity of the spermine-binding protein is also sensitive to actynomicin D administration. In fact the two fold increase of binding activity found 1.5 hr after 1,25-$(OH)_2D_3$ injection to rachitic chicks (panel A) was almost completely prevented when the animals received a prior injection of 50 μg of actynomicin D (panel B). The cycloheximide and actynomicin D sensitivity suggests that 1,25-$(OH)_2D_3$ in-

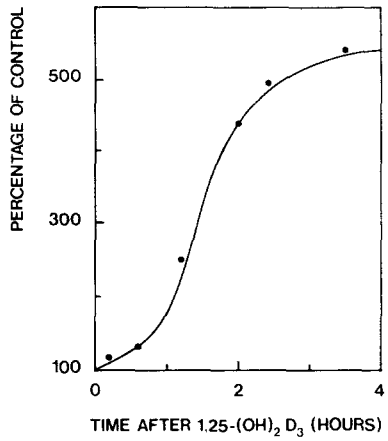

Fig. 2: Rapid effect of 1,25-$(OH)_2D_3$ on the spermine-binding activity of the duodenal protein fraction. Groups of rachitic chicks were dosed intracardially with 140 ng of 1,25-$(OH)_2D_3$ or vehicle and killed at the time indicated. The DEAE-cellulose chromatographed binding proteins were prepared from each group as described under the Methods section. Aliquots (150 µg) of each protein sample were pretreated with RNAse as described in the legend to fig. 1 and then assayed for their spermine-binding activities by the gel filtration method as described under the Methods section. The radioactivity of the spermine-protein complex eluted with the void volume of the effluent was calculated and compared, the result for the binding protein from vehicle injected rachitic chicks being taken as 100%. Each point represents the mean of at least four determinations.

duces the spermine-binding protein in the duodenal mucosa of vitamin D-deficient chick. However, it is also possible that 1,25-$(OH)_2D_3$ is able to induce other proteins or enzyme activities which, in turn, modify the binding protein and therefore enhance its spermine-binding activity. One of the several possibilities is phosphorylation. To examine this possibility we performed *in vitro* phosphorylation and dephosphorylation experiments using purified protein preparations from both rachitic and 1,25-$(OH)_2D_3$ injected animals. Spermine-binding activities are insensitive to alkaline phosphatase treatment and/or to phosphorylation by cAMP-dependent beef heart protein kinase, indicating that probably the phosphorylation status of the protein is not important for the control of the spermine-binding activity.

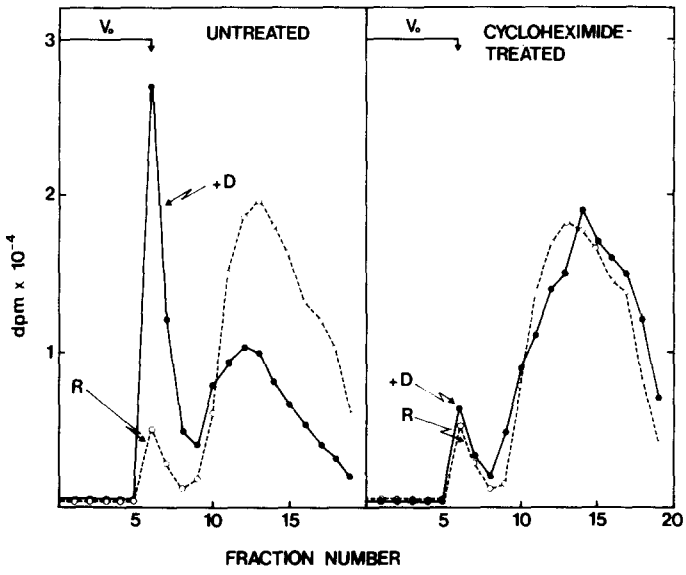

Fig. 3: Effect of cycloheximide on 1,25-$(OH)_2D_3$ stimulation of the duodenal spermine binding protein. Groups of rachitic chicks were injected intraperitoneally with or without 50 µg of cycloheximide in saline 2 and 4 hrs before to be killed. They were divided into two groups and injected without (R) or with 140 ng of 1,25-$(OH)_2D_3$ (+D) 2.5 hr before to be killed. The DEAE-cellulose chromatographed binding proteins were prepared from each group as described under the Methods section. Aliquots (170 µg) of each protein sample were pretreated with RNAse as described in the legend to fig. 1 and then assayed for their spermine binding activity by the gel filtration method as described under the Methods section.

We have also examined the possibility that the active form of vitamin D could modify the binding capacity of spermine-binding protein. In rachitic chicks before and after dosing with 1,25-$(OH)_2D_3$, the molar ratio of spermine to binding protein is 1:1 (unpublished results) as already shown in normal chicks (2).

From the above indirect evidence it appears that the active form of vitamin D is able to regulate the activity of the spermine-binding protein possibly by inducing the synthesis of new protein molecules. Direct evidence for a vitamin D-induced increase in binding protein will be obtained if our attempt to raise antibodies against this protein will be successful.

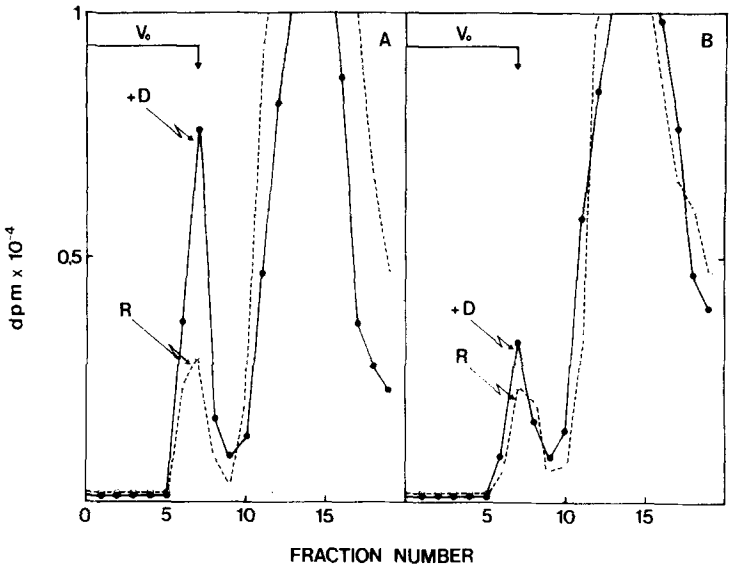

Fig. 4: Effect of actinomycin D on 1,25-$(OH)_2D_3$ stimulation of the duodenal spermine-binding protein. Groups of rachitic chicks were injected intraperitoneally with (panel B) or without (panel A) 50 µg of actinomycin D in saline 2.5 hrs before they were killed. They were divided into two groups and injected without (R) or with 140 ng of 1,25-$(OH)_2D_3$ (+D) 1.5 hr before they were killed. The DEAE-cellulose chromatographed binding proteins were prepared from each group as described under the Methods section. Aliquots (110 µg) of each protein sample were pretreated with RNAse as described in the legend to fig. 1 and assayed for their spermine-binding activities by the gel filtration method as described under the Methods section.

However, the increase of spermine-binding activity appears to be one of the earliest events following intracardial injection of the active form of vitamin D.

We do not know yet the biological significance of the duodenal spermine-binding protein and of this rapid enhancement of binding activity. If this protein is an enzyme or a regulatory part of an enzyme, its activity may be regulated by spermine. Alternatively the polyamine-binding protein may play a major role in the intracellular communication of the hormone message since the protein and/or spermine may be translocated to specific cellular organelles such as ribosomes or chromatin to fulfill their structural or functional roles in mediating the response to 1,25-$(OH)_2D_3$ in the duodenal cell.

Polyamine-conjugated proteins have been detected in several tissues and fluids which may or may not be target for vitamin D or other steroids i.e. chick duodenal mucosa (1) rat ventral prostate (7) human amniotic fluid (8), human and rabbit serum (9, 10). Polyamines have also been shown to be released or taken up in relation to cell cycle (11). Therefore it is possible that different polyamine binding proteins play not only an intimate role in spermine compartmentalization within the cell or spermine conjugation outside the cell, as already suggested (10), but that the internal and external concentrations of the binding proteins directly correlate with the ability of the tissue to retain or release polyamine during the cell life.

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