Vol. 97, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 17, 1980 Pages 222-229

POLYAMINE BINDING BY A CYTOPLASMIC FACTOR IN THE DUODENAL MUCOSA OF NEW-BORN CHICK

G. Mezzetti, M.S. Moruzzi*, G. Capone and B. Barbiroli

Istituto di Chimica Biologica dell'Università di Modena,
Via Campi, 287 - 41100 Modena (Italy)

*Istituto di Chimica Biologica dell'Università di Bologna,
Via Irnerio, 48 - 40126 Bologna (Italy)

Received September 24,1980

SUMMARY

The incubation of micromolar concentrations of radioactive spermine with cytosol from chick duodenum leads to the formation of a non-covalent binding between spermine and a macromolecular component of the cytosol. This component seems to be a protein since trypsin but not ribonucleases can abolish the formation of the complex. The spermine-binding activity is also present in the duodenum of 18-day old chick embryo, but is dramatically lower when compared to the duodenal factor of a new-born chick.

INTRODUCTION

Selective effects of polyamines on bacterial and mammalian cells have been extensively described. Polyamines have been found to have major effects on DNA structure and synthesis (1), enzyme activity (2) as well as in promoting forms of transcription and translation characteristic of *in vivo* systems (3, 4). Since polyamines may interact with cellular proteins it is possible that some of their biological effects may be ascribed to this interaction. A naturally occurring spermine-binding protein has been identified in normal rabbit serum and it has been proposed to have a specific role as conjugator for spermine molecules released from the cells (5).

In this paper we report that chick duodenum, a polyamine-rich tissue, and sensitive to the steroid 1,25-$(OH)_2D_3$*, contains a

*Abbreviation: 1,25-$(OH)_2D_3$ = 1,25-dihydroxy vitamin D_3.

0006-291X/80/210222-08$01.00/0

protein(s) capable of binding spermine with high affinity. The spermine-binding protein is present in the duodenal cell of 18-day old chick embryo and its activity is remarkably increased after hatching.

MATERIALS AND METHODS

Chemicals. [^{3}H] Spermine tetrahydrochloride (44.3 Ci/mmole) and other radioactive polyamines were obtained from New England Nuclear. Electrophoretically pure nucleases and crystalline trypsin, obtained from Worthington, were always desalted before use. Whole tRNAs from rat liver was obtained from General Biochemicals. Animals used in all experiments were one-day old White Leghorn chicks obtained from a commercial hatchery.

All manipulations were carried out at 0-2°C. The duodenum was removed, rinsed in 40 mM glycine buffer, pH 8.7, 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 one hour. The upper three quarters of the supernatant were taken and used as the cytosol fraction. Solid $(NH_4)_2SO_4$ was added slowly into the cytosol fraction with stirring until the salt concentration reached 55% of saturation, and the precipitate formed after 30 min stirring was removed by centrifugation. The supernatant was then made to 100% of saturation with respect to $(NH_4)_2SO_4$. The precipitate formed after 4 h stirring was collected by centrifugation, resuspended in 40 mM glycine buffer, pH 8.7, and dialyzed extensively against 2 liters of the same buffer.

The protein sample was incubated with [^{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 measurement of the amount of radioactive spermine bound to the macromolecules in the void volume of the effluent.

The radioactivity was measured by dissolving an aliquot of aqueous sample in Toluene-Triton-X-100 (3:1), 0.4% w/v 2,5-diphenyloxazole, 0.005% w/v, 1,4-bis-2-(5-phenyloxazolyl)-benzene, and 5% H_2O scintillation liquid. Protein was measured by the method of Warburg and Christian (6). In the affinity chromatography experiments, spermine was bound covalently to Sepharose as described by Castañeda and Liao (7). The apparent molecular weight of the binding factor was estimated, after fixing the radioactive spermine with glutaraldehyde, by disc gel electrophoresis on 12.5% acrylamide, 1% sodium dodecyl-sulfate according to Mezzetti et al. (8). The standard markers used in the estimation of the molecular weight of the binding protein and

their molecular weights were: ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), α-lactalbumin (14,400).

RESULTS AND DISCUSSION

The results presented in this paper show that chick duodenum, a polyamine rich tissue, does contain a spermine binding protein(s). As shown in Fig. 1A, when crude cytosol from chick duodenum is incubated with [^{3}H] spermine and chromatographed on a Sephadex G-25 column a sharp radioactive peak was eluted in the void volume of the effluent. This fact has been interpreted as due to an association between the radioactive polyamine and a macromolecular component(s) of the cytosol.

The macromolecular polyamine binder is resistant to extensive treatment with ribonucleases. The RNAse solution utilized in the experiment depicted in Fig. 1 was a mixture of pancreatic RNAse A and RNAse T_1. Fig. 1B shows the effectiveness of the RNAse digestion of rat liver tRNA in abolishing the radioactive peak in the void volume of the effluent. On the other hand the polyamine binder is sensitive to trypsin digestion. In Fig. 1C is reported the binding activity of 1 mg of duodenal cytosol protein pretreated with RNAses and then incubated for 4 h at 25°C with 150 μg of trypsin. This treatment is effective in abolishing all the RNAse resistant radioactivity eluted in the void volume of the effluent.

The binding between spermine and duodenal factor was completely prevented by heating the protein preparation at 90°C for 15 min. When 2 M KCl or other monovalent or divalent cations were added to the assay mixture the radioactive ligand was dissociated from the complex suggesting the non-covalent nature of the binding. Because of the tight binding of spermine to the test tube wall (9) an accurate measurement of the affinity constant of the spermine-protein complex was not possible. For this reason, each experiment was evaluated individually and only the relative binding activities were compared. With increasing concen-

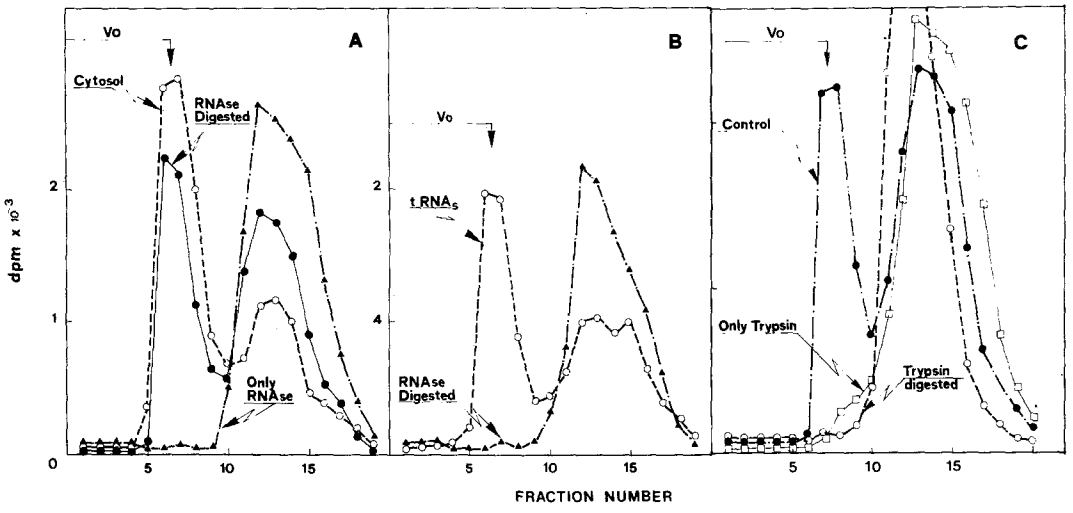


Fig. 1: Radioactive spermine-binding by a RNAse-resistant and trypsin-sensitive factor from chick duodenum cytosol. Panel A: 0.1 μCi of 4 μM [^{3}H] spermine was incubated with 1 mg of dialyzed cytosol protein undigested with RNAse (o--o) or digested with 50 μg of a RNAse mixture (pancreatic + T_1, 20:1) for 20 min at 25°C (●—●), or in the absence of cytosol protein (▲-·-▲). Panel B: pure rat liver tRNAs (3 A_{260}) replaced cytosol protein. Panel C: 0.1 μCi of 4.7 μM [^{3}H] spermine was incubated with 1 mg of dialyzed cytosol protein pretreated with RNAse, untreated with trypsin (●-·-●); or treated with 150 μg of trypsin for 4 h at 25°C (o--o); or in absence of cytosol protein (□—□). The mixtures were analyzed by gel filtration as described under Materials and Methods.

trations of cold spermine in the test system the amount of [^{3}H]-spermine bound decreased as expected if a binding substance was approaching saturation (Fig. 2). The protein factor(s) is quantitatively precipitated from the cytosol preparation by addition of ammonium sulfate to 55-100% of saturation. Further purification of the spermine-binding factor(s) was achieved by Sephadex G-100 gel filtration, spermine-Sepharose affinity column and polyacrylamide gel electrophoresis, but so far, a large-scale purification of the protein has not been carried out. The apparent molecular weight of the binding protein estimated by sodium dodecyl-sulfate disc gel electrophoresis, after fixing of the radioactive spermine to it by glutaraldehyde, was about 32,000. To test the relative polyamine or diamine specificity

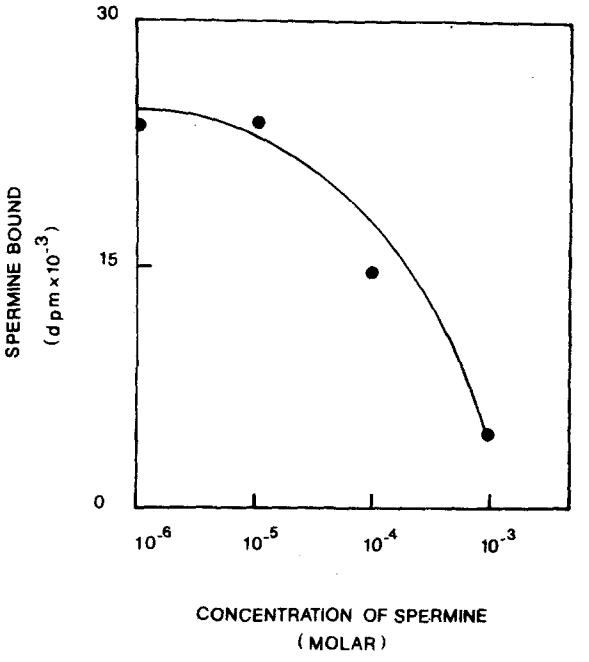
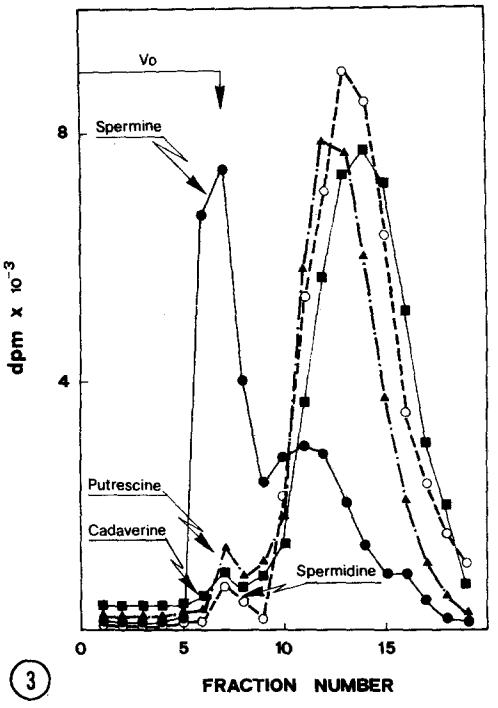


Fig. 2: Competition of [^{3}H] spermine binding by nonradioactive spermine. 1.8 mg of dialyzed cytosol protein pretreated with RNAse as described in the legend to Fig. 1 was incubated with 0.17 μCi of 1 μM [^{3}H] spermine in the absence or in the presence of various concentrations of nonradioactive spermine. The mixtures were analyzed by gel filtration as described under Materials and Methods.

of the duodenal binding factor, radioactive spermine, spermidine and putrescine were compared for their binding to the protein (Fig. 3). At 10 μM concentration of these amines 1 mg of the 55-100% ammonium sulfate fraction protein preincubated with RNases could form a large distinct complex peak only with spermine. The incubation with putrescine, spermidine or cadaverine resulted in a much less binding frequently near the limit of detectability.

In several publications since 1966 Wasserman and co-workers (10-12) reported the isolation and characteristics of a vitamin D-dependent calcium-binding protein in different animal species and its relation to intestinal absorption of calcium. The calcium-binding protein has several characteristics similar to the



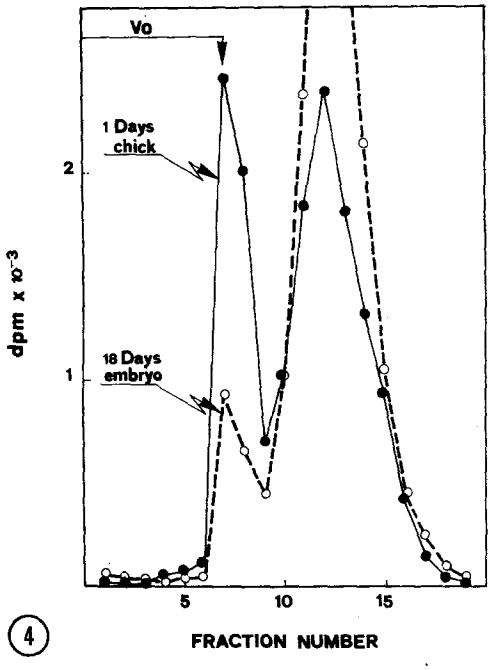


Fig. 3: Binding of radioactive polyamines to the proteins of 55-100% ammonium sulfate fraction of the duodenal cytosol. 1.2 mg of protein from the 55-100% ammonium sulfate fraction pretreated with RNAse as described in the legend to Fig. 1 was incubated with 0.15 µCi of the indicated polyamines at the final concentration of 10 µM. The mixtures were analyzed by gel filtration as described under Materials and Methods.

Fig. 4: Spermine-binding activity in the cytosol of duodenum of one-day old chick and 18-day old chick embryo. 0.1 µCi of 6 µM [^{3}H]spermine was incubated with 1 mg of cytosol protein pretreated with 50 µg of RNAse solution as described in Fig. 1. The mixtures were analyzed by gel filtration as described under Materials and Methods.

spermine-binding one. It is a low molecular weight protein, present in the same ammonium sulfate fraction, and it is an acidic protein as one can expect to be a polyamine binding protein. However some physical characteristics between the two factors are clearly different and from the competition experiments previously reported (13) it appears that in the duodenal cells different factors bind selectively calcium and spermine. In any case final proof will be given immunologically and/or

from competition studies using pure calcium-binding protein and spermine-binding factor.

The protein described here, in certain features, resembles the androgen-sensitive (8) spermine-binding protein from rat prostate reported by Liang et al. (9). To have some indication on the physiology of the duodenal spermine-binding protein we measured its activity in the chick embryo and in the new-born chick (Fig. 4). The spermine-binding protein is present in the duodenal cell of the new-born chick as well as of the 18-day old embryo, but its activity is dramatically higher after hatching. At the moment we do not know yet whether the increased polyamine binding activity observed in the duodenum of one-day old chick is due to a synthesis of new molecules of protein, or to an activation of preexistent molecules of a less active factor.

Studies are in progress to ascertain if the active form of vitamin D is directly involved in the regulation of the duodenal spermine-binding activity. Finally the possibility that this binding factor may be physiologically correlated with the intracellular transport and localization of spermine in the duodenal cell will be considered.

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