RADIOIMMUNOASSAY FOR A VITAMIN-D DEPENDENT CALCIUM-BINDING PROTEIN IN RAT DUODENAL MUCOSA

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SUMMARY. A radioimmunoassay for a vitamin D-stimulated calcium-binding protein from rat intestine has been developed. The assay can measure as little as 0.5 ng protein and has allowed to detect an immunoreactive material in rat serum. On the other hand, no cross-reactivity has been observed with either the protein from rat kidney or that from pig duodenum.

1 - INTRODUCTION. Vitamin-D dependent calcium-binding proteins† (CaBP) involved in the intestinal calcium transport have been identified and characterized in the duodenal mucosa from many species (1). To understand the physiological role played by intestinal CaBPs in the mechanism of action of vitamin-D, one has first to quantify the CaBP concentration in tissue extracts or biological fluids. Quantitation has been carried out by radioimmunoassay for pig CaBP (2) and by immunodiffusion assays for beef and chick CaBP (3,4). Obtention of anti-CaBP antibodies for rat CaBP has not so far been reported, and the most commonly used method for assay of rat intestinal CaBP has depended on the competition of this protein with a calcium-chelating resin (Chelex) (5,6). This method, in which measurement of CaBP is based upon its calcium-binding capacity, lacks sensitivity and specificity. Moreover it cannot be used in unpurified tissue extracts, since they contain too high a content of molecules capable of binding calcium either unspecifically or with low

†Abbreviations : CaBP, vitamin-D dependent calcium-binding protein ; BSA, bovine serum albumin ; PBS, phosphate buffered saline.

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affinity. These results have led us to develop a radioimmunoassay for rat intestinal CaBP. In this paper we report the production of anti-CaBP antibodies and the preparation of a radioactive iodinated protein used as tracer in the assay. Sensitivity, specificity and validity of the assay are discussed. CaBP concentrations in duodenal mucosa of rats under various pathophysiological conditions are also presented.

2 - MATERIALS AND METHODS. All experiments were carried out at 4° C unless otherwise stated. Chelex 100 resin was purchased from Bio Rad (USA) and $^{45}CaCl_2$ from CEA (France). Lactoperoxidase and bovine serum albumin (BSA) came from Sigma (USA) and carrier free Na-^{125}I from the Radiochemical Center (Amersham). Polyethylene glycol 6000 was from Fluka (Switzerland). All other chemicals were purchased from Prolabo (France).

2-1-Purification of CaBP. Partially purified CaBP from rat duodenal mucosa was first prepared according to Freund and Bronner's technique (6). Taking advantage of its Ca-binding properties, the protein was then purified by successive gel filtration and elution on DEAE Sephadex anion exchange resin as for pig duodenal CaBP (7). Protein concentrations were measured by a modified Lowry procedure (8). Quantitative and qualitative calcium-binding activity were determined as described by Freund and Bronner (6). Analytical gel electrophoresis was carried out according to the method of Davis (9). Purified CaBP was divided in small amounts, lyophilized and stored at -30° C. Such preparations were used i) for immunization of rabbits, ii) for preparing iodinated CaBP and iii) as reference for the standard curves of the radioimmunoassay (see below).

2-2-Preparation of antisera. Five adult male white rabbits were immunized intradermally with an emulsion of CaBP (100 µg) and complete Freund's adjuvant. Bleedings were taken twice a month and tested for antibody binding parameters.

2-3-Iodinated tracer preparation. Iodinated CaBP was prepared at room temperature by enzymatic iodination using lactoperoxidase (10). To 10 µl (4 µg) of CaBP, successively 5 µl of PBS 0.5 M pH 7.4, 10 µl of Na-^{125}I (∿1mCi), 5 µl (5 µg) of lactoperoxidase (in PBS 0.05 M pH 7.4) and 1 µlH_2O_2 (0.88mM) were added. This mixture was stirred for 30 s and again 1 µlH_2O_2 (0.88 mM) was added each 30 s three times. The mixture was stirred for 2 min before addition of 400 µl PBS 0.5 M to stop the reaction. The iodinated mixture was filtered through a Sephadex G 25 column (h = 25 cm, Ø = 1.5 cm, equilibrated and eluted with Tris-HCl 50 mM, BSA 0.5 % pH 7.4) to remove iodide. The peak eluted with the void volume was loaded onto a Sephadex G 75 column (h = 100 cm, Ø = 1.5 cm, equilibrated and eluted with the same Tris-HCl buffer) to purify ^{125}I-CaBP. The labelled protein was detected by gamma counting and by its elution volume (Ve = 2.2 Vo) which was similar to that observed with the purified unlabelled protein. This radioactive material was stored at -30° C for further immunological tests.

2-4-Standard radioimmunoassay procedure. BSA (0.5 %) containing PBS (0.02 M pH 7.4) was routinely used to dilute the reagents of the assay. Iodinated CaBP (0.1 ml, i.e. 12000 dpm, ∿100pg) and

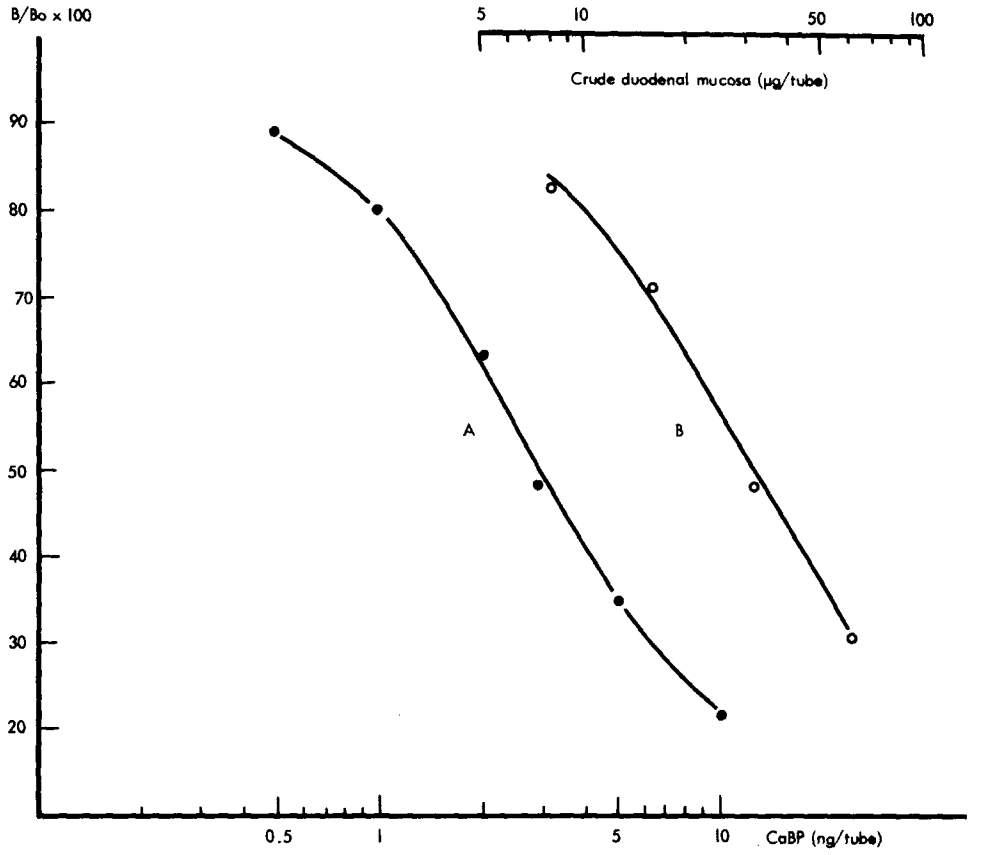

Figure 1. Dose response curves for rat duodenal CaBP. Curve A : Inhibition of ^{125}I-CaBP binding to anti-CaBP serum in the presence of increasing amounts of unlabelled CaBP. Curve B : Dilution curve obtained with a 100,000 g supernatant originating from duodenal mucosa of 5 week old hypocalcemic rats (Sprague strain) fed with a vitamin-D deficient diet. B and B_o are the precipitated radioactivity in the presence and absence of unlabelled CaBP, respectively.

dilute antiserum (0.1 ml) were successively added to 0.1 ml standard CaBP or buffer in polypropylene test tubes. The mixture was incubated overnight at 4° C. To precipitate antibody-bound proteins, a volume of 0.5 ml of polyethyleneglycol 6000 (11) (11 % final w/v) was added to each tube followed by 0.1 ml of bovine gamma globulin solution (0.5 % in PBS). The tubes were immediately centrifuged at 2,200 g for 20 min and the radioactivity of the precipitate was counted.

3 - RESULTS AND DISCUSSION. Purified CaBP exhibited Ca-binding parameters similar to those reported by Freund and Bronner (6) i.e. two high affinity (10^6 M^{-1}) calcium sites per molecule

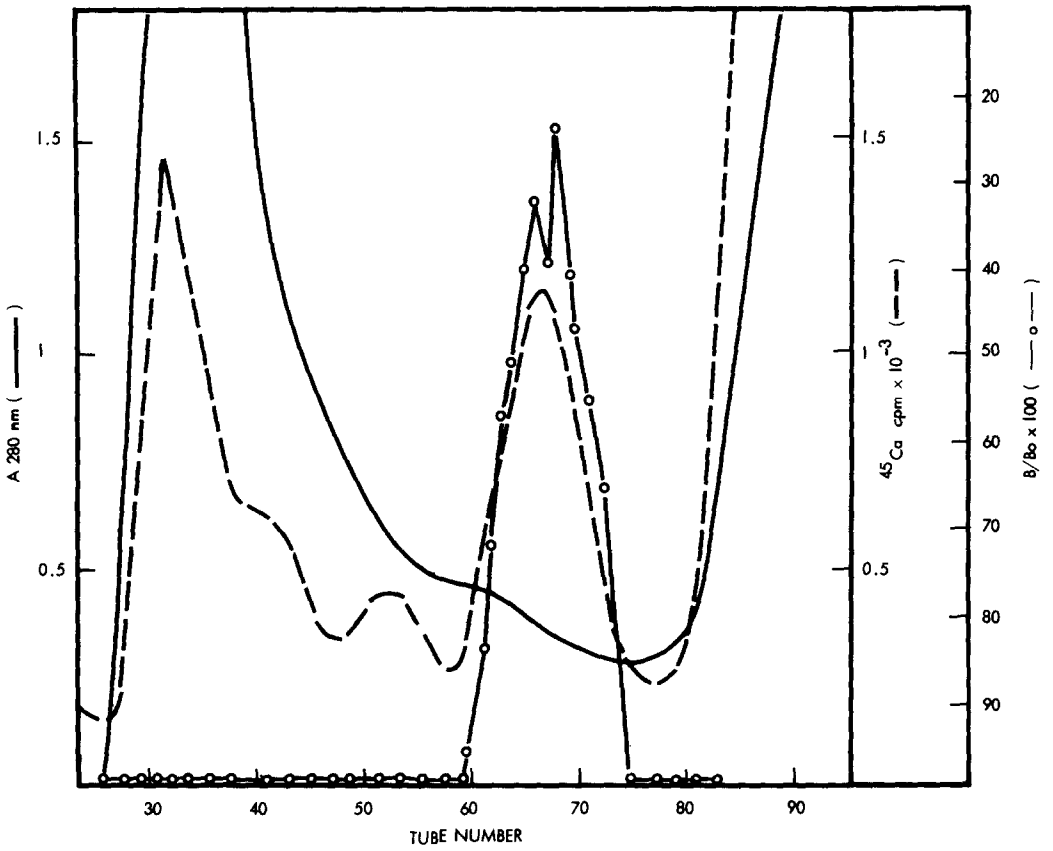

Figure 2. Sephadex G 75 gel filtration of a 100,000 g supernatant of rat duodenal mucosa. The column (100 x 1.5 cm) was eluted with ammonium acetate buffer, 20 mM, pH 7.2. Each fraction (4.5 ml) was tested for its Ca-binding capacity (------), measured by the Chelex method (6), and for its immunoreactivity (——o——).

(MW∿10-12000). A 10 % polyacrylamide gel electrophoresis of this material showed two protein bands which have been reported to possess similar specific Ca-binding activity (6). Fullmer and Wasserman (3) have also reported that bovine intestinal CaBP exhibited two protein bands, immunologically identical.

Two months after initial immunization, several bleedings showed a positive response for binding of the labelled protein. For the best blood samples the antiserum dilution required to obtain 40-50 % binding (B_o) with the iodinated tracer, was 1/6000 to 1/10000. It was also demonstrated that a 5 hour incubation at

4° C was necessary to reach equilibrium of the antigen-antibody reaction. Moreover, in the absence of antibody, only 5-6 % of radioactivity was precipitated with the polyethylene glycol technique ; no improvement of the binding parameters was obtained when using the double antibody precipitation technique. In addition, no Ca^{2+} concentration dependence (between 3.5 10^{-5} and 3.5 10^{-3} M) of the complex formation was observed.

Figure 1 illustrates a typical standard curve for measuring CaBP (curve A). When logit transformation (12) was used, a linear regression was obtained from 0.5 to 10 ng, the mean slope of which was - 1.23 $\pm$ 0.08 SE. Thus, the sensitivity of the radioimmunoassay was such that as little as 0.5 ng CaBP could be measured.

Specificity of the antibodies has also been verified. We have tested the immunoreactivity of each fraction eluted from a Sephadex G 75 column on which a 100,000 g supernatant of homogenized rat duodenal mucosa had been loaded. Although some Ca-binding activity appears associated with proteins of high molecular weight, figure 2 shows that a single immunoreactive peak was obtained which was superimposable with the vitamin D-dependent CaBP peak, i.e. that eluted at Ve = 2.2 Vo. We have also observed that 16 µg of renal cortex proteins (issued from a 100,000 g supernatant of this homogenized tissue) was required to inhibit the ^{125}I-CaBP binding to antiduodenal CaBP to the same extent as that produced by 50 ng of duodenal proteins (from a similar issue). Since renal CaBP has been reported to have a molecular weight of about 28000 (13), these results require further investigation as is the case for pig CaBPs, where similar findings have been published (14). The absence of cross-reactivity between rat and pig duodenal CaBP has also been demonstrated ; adding up to 700 ng of pig CaBP (as determined by the Chelex method) had no effect upon the antigen-antibody complex. In the case of the pig duodenal CaBP radioimmunoassay, no cross-reactivity with CaBP from rat duodenum was observed (14).

Validity of the assay has also been studied. In a first set of experiments, the CaBP content in supernatant of homogenized duodenal mucosa (100,000 g) was determined (Figure 1) : the parallelism between the curve obtained and that corresponding to

standard CaBP suggests that the two materials are immunologically identical. On the other hand, as expected, similar results have been obtained with partially purified CaBP preparations (e.g. fractions 60-73 of figure 2). With such preparations CaBP concentrations determined by the assay agree perfectly with those obtained via the Chelex method. Immunoreactive CaBP has also been found in serum. The serum immunoreactivity was parallel to that of pure CaBP, as in the case of pig CaBP (14).

Furthermore, data obtained with hypocalcemic rats have shown that the CaBP content of vitamin-D deficient animals was 73-90 µg/g mucosa whereas in animals supplemented with 5 and 10 i.u. of 25-hydroxycholecalciferol (doses were given by a single intravenous injection, 48 hours before sacrifice) the CaBP levels rose to 1380 and 1660 µg/g, respectively. CaBP concentrations in the serum of the two latter groups of animals were found to be 16 and 20 ng/ml, respectively. These results clearly confirm the vitamin-D dependence of the CaBP studied.

The assay for CaBP reported here is 600 times more sensitive than the Chelex method which can detect a minimum concentration of CaBP of about 3 µg/ml. Moreover it can be used to measure directly the CaBP content of tissue extracts and biological fluids such as serum.

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