Control of Vitamin D-dependent Calcium-binding Protein in Rat Intestine by Growth and Fasting*

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The vitamin D-dependent calcium binding protein (CaBP) content of the proximal small intestine of normal female rats was related to the animal's growth rate and feeding pattern. CaBP in a low molecular weight fraction of 40,000 x g duodenal supernatant was quantitated by 45Ca saturation analysis; changes in CaBP were confirmed by polyacrylamide disc gel electrophoresis with the use of a purified CaBP marker. The total amount of CaBP was unchanged between 4 and 9 weeks of age during the rapid phase of growth. By 13 and 20 weeks of age, when the growth rate had decreased to less than 10 g/week, CaBP levels were 16% and 30%, respectively, of values obtained in the younger animals. These decreases in CaBP with age were inversely related to changes in total mucosal protein content. The amount of CaBP/g of body weight decreased between 4 and 20 weeks of age in an exponential manner and paralleled the exponential decay in growth rate. At all ages, CaBP decreased by an average of 58% following a 22-h fast, whereas total mucosal protein decreased by only 16%. These alterations in CaBP are consistent with previously reported changes in calcium absorption observed in both well fed and undernourished animals. The data indicate that the vitamin D-dependent CaBP is affected by undefined factor(s) which condition the response to both growth and fasting.

A rapid and simple method is described for isolating rat intestinal vitamin D-dependent CaBP in milligram quantities; the purified CaBP (M, = 10,000) has high affinity sites for Ca2+ binding, an apparent dissociation constant of 0.3 μM, with a maximum binding capacity of approximately 0.21 μmol of Ca2+/mg of protein (~2 mol of Ca2+/mol of CaBP).

Numerous studies suggest a close correlation between intestinal calcium absorption and a duodenal calcium-binding protein which is vitamin D4 dependent (1). Direct relationships of CaBP levels have been reported with in vivo alterations in calcium absorption induced by vitamin D deficiency (2, 3), age (4, 5), egg laying (4, 6), dietary calcium and phosphorus (4, 7-9), strontium (10), dilantin (11), and Solarium malacoxylin (12). In most of these situations, alterations in the levels of vitamin D metabolites can explain the changes in both CaBP and calcium absorption (13). An exception appears to be the effect of aging in which alterations of vitamin D metabolism have not yet been defined.

The present report is the first characterization of the effects of fasting on CaBP, and of CaBP levels during the period of rapid growth. Earlier studies by others have related CaBP to dietary calcium intake. Under normal feeding conditions, low calcium intake results in increased levels of intestinal CaBP (4, 7-9). However, in the present study, acute fasting with a loss of 10 to 16% body weight produced a marked decrease in rat duodenal CaBP. Previous studies on aging and intestinal CaBP have compared only old animals to young animals, specifically young chicks to hens (4), and 100-g rats to animals weighing over 500 g (5). The results of this report indicate that the level of CaBP is tailored to the growth rate of the animal and is predictable for a given growth rate. These observations are consistent with the hypothesis that a vitamin D-dependent intestinal CaBP may prove essential to ensure maximal skeletal growth (1).

MATERIALS AND METHODS

45CaCl2 was purchased from New England Nuclear at a specific activity of 15 to 20 mCi/mg. Chelex 100 (200 to 325 mesh, sodium form) was obtained from Bio-Rad. All polyacrylamide gel reagents and disc gel equipment were ordered from Canadeco, Inc. Tris base was a product of Sigma. The slab gel was purchased from E-C Apparatus Co. Sephadex G-100 was purchased from Pharmacia. Glass beads (6 mm) were ordered from Fisher and UM-05 filters were obtained from Amicon. Female rats were obtained from Holtzman Co. and maintained on Ralston Purina rat chow (1.2% calcium, 0.8% phosphorus) for at least 1 week before any experiment. All animals were housed in wire mesh cages and had free access to water. In the ad libitum experiments, the rats had ample food in their cages at all times. In the fasting experiments, food was removed from cages at 5:00 p.m. on the day prior to being killed. In all experiments the animals were killed between 2:00 and 3:00 p.m. on the following day.

Calcium Binding Assay — CaBP activity was quantitated by the calcium binding capacity in a 45Ca/Chelex resin assay (14-16). Plastic reagent bottles, disposable test tubes, and pipette tips were used to minimize Ca2+ contamination. The Chelex resin was washed extensively with HCl and equilibrated in Buffer A (10 mM Tris/HCl, 5 mM 2-mercaptoethanol, pH 7.2). The amount of contaminating Ca2+ in the resin, buffer, and protein solutions was determined by atomic absorption spectrophotometry (17). Assays were performed at 20°C in a final volume of 0.325 ml which contained resin, 70 to 200 μg of protein, 0.1 μCi of 45CaCl2, and added CaCl2, the total calcium, unless otherwise noted, varied between 7 x 10−4 M to 1 x 10−2 M. The
resin was ultimately sedimented by centrifugation at 20,000 g. Bound calcium was determined by liquid scintillation counting of 25 μl of supernatant aliquots. The results were expressed as a double reciprocal plot of Ca²⁺ bound/mg of protein and free Ca⁺⁺ (8); the binding maxima (nmol of Ca²⁺ bound/mg of protein) and apparent dissociation constants were calculated by means of a Hewlett-Packard calculator and least squares fitting of the data. "Units" of calcium binding activity were defined as maximum binding capacity ("specific activity") multiplied by the total protein (milligrams) contained in a fraction.

Isolation and Purification of Intestinal CaBP — The vitamin D-dependent intestinal CaBP was isolated from intestinal extracts by modification of the methods of Kalkuhl et al. (17) and Dresher and DeLuca (18). The animals (three to four rats per group) were killed and the proximal small intestine (12 cm from the pyloric valve) was excised. All subsequent procedures were performed at 4°C. The intestinal binding was rinsed with approximately 6 ml of Buffer A. The flow rate was 15 ml/h and 2-ml fractions were collected. The fractions were assayed for calcium binding activity by means of the ⁴⁰Ca/Chelex partition method at an added Ca⁺⁺ concentration of 13 ELM. Chromatographic fractions (tubes 34 to 43) which contained the 5,000 to 20,000 molecular weight CaBP activity were pooled and designated Fraction II. The latter was concentrated 50-fold by Amicon ultrafiltration with UM-05 filter and the sample prepared for slab gel electrophoresis by adding sucrose to 0.25 to 0.30 (Rₛ = 0.25 to 0.30) was pooled and designated Fraction III. In order to remove the Ca gel from Fraction III, the fraction was either subjected to ultracentrifugation (Amicon UM-05) or concentrated and chromatographed on a Sephadex G-25 column equilibrated in Buffer A. The purified Fraction III sample was lyophilized and stored at -70°C.

RESULTS

Purification and Characterization of Rat CaBP — The basic preparation and purification of intestinal CaBP as outlined under "Materials and Methods" involved three steps which are summarized in Table I. The 40,000 x g supernatant fraction of mucosal homogenates was rapidly chromatographed by a glass bead/Sephadex G-100 technique (19). The low molecular weight CaBP fraction was pooled and further purified by slab gel electrophoresis. As noted in Fig. 1, the peak of CaBP activity consistently migrated with an Rₛ between 0.25 and 0.30. Minor peaks of slower (Rₛ = 0.15 to 0.22) or faster (Rₛ = 0.32 to 0.36) mobility were occasionally observed. The CaBP activity of Fraction III was estimated to be 10,000 molecular weight by means of the gel filtration techniques described under "Materials and Methods." The mean (± S.E.) specific activity of Fraction III obtained from five separate preparations of CaBP was 0.24 (± 0.04) nmol of Ca⁺⁺ bound/mg of protein. This corresponds to the binding of approximately 2 mol of Ca⁺⁺/mol of protein based on the protein's molecular weight of 10,000. An apparent dissociation constant for Ca⁺⁺ binding, Kₛ, was estimated by a double reciprocal plot (Fig. 2), and the mean value (± S.E.) from five preparations was 0.3 (± 0.1) μM. Fig. 3 illustrates the protein staining patterns of Fractions II and III after polyacrylamide disc gel electrophoresis. Measurements of CaBP activity of sequential segments of the slab gel (Fig. 1) revealed that the CaBP activity of Fraction II increased between segments 1 and 9 while that of Fraction III was less active in the distal small intestine.

TABLE I

Purification of rat intestinal low molecular weight CaBP

| Fraction | Supernatant | G-100 | Slab gel |
|----------|-------------|------|---------|
|          | (40,000 x g)|      |         |
| Fraction I | 488          | 1862 | 3.42 100 |
| Fraction II | 50,000 x g | 28.6 | 752 26.3 |
|           | (Mₛ = 0.25) | 26.3 | 45.2  |
| Fraction III | 2.1          | 441  | 210 26.5 |
| Slab gel (Rₛ = 0.25-0.30) | 210 26.5 |

Fig. 1. Slab gel electrophoresis of Fraction II. Approximately 25 mg of protein was applied per slab; electrophoresis and elution of protein were performed as described under "Materials and Methods." The CaBP activity (••••••) was expressed as the percentage of binding of ⁴⁰Ca in supernatant with the Chelex method as outlined under "Materials and Methods." The ⁴⁰Ca binding activity between 0.25 and 0.30 Rₛ was pooled and designated Fraction III. The protein pattern of Fraction II is indicated by Coomassie blue staining of a slab gel slice.
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Effect of Age on Proximal Small Intestinal Proteins and CaBP in Fed Rats - Table II summarizes the normal growth pattern of female rats fed ad libitum from 4 to 20 weeks of age. A rapid growth phase was noted between 4 and 9 weeks of age (81 to 201 g) followed by a slower rate of weight gain between 9 and 20 weeks of age (201 to 274 g). Although the total body weight greatly changed between 4 and 9 weeks of age, changes in total mucosal protein and Fraction I protein from the proximal small intestinal segment were insignificant. Progressive maturation was associated with small increases in protein content. By 20 weeks of age, 30% and 55% increases in protein content were observed in total mucosal protein and Fraction I protein, respectively.

Age-related changes observed in the protein content of the low molecular weight Fraction II more closely paralleled the total body weight of the animals. Comparing all fed animals, a 2-fold increase in Fraction II was detected from 4 to 9 weeks of age, although no further increments were noted at 15 and 20 weeks of age.

The CaBP pattern was strikingly different from that of other intestinal protein fractions. Total CaBP activity in Fraction II was constant between 4 and 9 weeks of age. However, by 15 weeks of age there was a 54% decrease in CaBP activity observed at 4 to 9 weeks of age, and by 20 weeks of age the activity was only 30% of that observed in the youngest animals. The age-related quantitative decrease in CaBP was confirmed by polyacrylamide disc gel analysis (Fig. 4). When constant amounts of low molecular weight protein (Fraction II) were applied to each gel, the specific activity of CaBP in this fraction paralleled the percentage of the CaBP band in disc gel. At 4, 9, and 15 weeks of age, the mean (± S.E.) specific activity of CaBP (nmol of Ca2+ bound/mg of protein) of Fraction II (Table II) was 21.4 (±1.4), 11.8 (±2.1), and 5.0 (±0.6), respectively.

When CaBP of intestinal segments, expressed in terms of body weight, was analyzed as a function of age (Fig. 5), an exponential decrease in CaBP was apparent between 4 and 20 weeks of age which was similar to the declining growth rate of normal rats (28). In fact, duodenal CaBP content per g of body weight proved to be a direct linear function of the rat's growth rate as shown in the inset of Fig. 5. Similar analyses of other proteins will be presented elsewhere.

Fig. 2. Binding of calcium to Fraction III at various concentrations of calcium in the assay (including added Ca2+ and endogenous Ca2+ from protein, buffer, and Chelex) which varied between 2.7 × 10^-5 M and 4.0 × 10^-4 M. The free Ca2+ (2.4 × 10^-8 M to 2.6 × 10^-7 M) was estimated as described under "Materials and Methods." The inset depicts a double reciprocal plot of bound calcium (nmol of Ca2+/mg of protein) versus free Ca2+ (M).

Fig. 3 (left). Polyacrylamide disc gels were performed as described under "Materials and Methods." A, Fraction II, 15 µg of protein with ~3.0 × 10^-5 M Ca2+; B, Fraction III, 2.5 µg of protein with ~4.5 × 10^-5 M Ca2+; C, Fraction III, same as B except with the addition of 0.1 mM EDTA in the electrode buffer. The arrows define the direction of migration.

Fig. 4 (right). Polyacrylamide disc gel electrophoresis of the low molecular weight intestinal CaBP (Fraction II of 4-, 9-, and 15-week-old fed rats. Each gel had ~6.8 µg of protein. Electrophoresis in EDTA and staining of the proteins were performed as described under "Materials and Methods." The direction of migration was from top to bottom. The arrow designates the CaBP band as determined from Rf properties of purified CaBP in an EDTA buffer system (Fig. 3).

Fig. 5. Comparison between units of CaBP (nmol of Ca2+ bound in Fraction II) per g of body weight vs. age (weeks) with the growth rate (weight gain (g) per week) vs. age (weeks). The growth rate at each age represents the total gram increase in weight for the week preceding the experiment. The inset illustrates the direct relationship between growth rate (grams per week) and CaBP units/g of body weight.
TABLE II
Effects of age and fasting on protein and CaBP content of rat proximal small intestinal segment (12 cm)

Animal weight was determined before death. Protein and CaBP were measured as described under "Materials and Methods." The results are expressed as mean (± S.E.). The numbers in parentheses indicate the number of experiments, one animal per experiment except for Fraction II values of protein and CaBP measurements. In the latter cases, three to four rats were pooled for each experiment and the data calculated per duodenal segment.

| Age      | Weight (g) | Total homogenized mucosal protein (mg) | Supernatant (40,000 × g) protein (Fraction I) (mg) | Low molecular weight protein (Fraction II) total protein (mg) | CaBP units (Fraction II) (nmol Ca\(^{2+}\)) bound |
|----------|------------|----------------------------------------|--------------------------------------------------|---------------------------------------------------------------|--------------------------------------------------|
|          | Fed        | Fasted                                 | Fed                                              | Fasted                                                       | Fed                                              |
| 4 weeks  | 81.3 ± 2.7 (15) | 68.4 ± 1.6 (14) | 45.8 ± 2.2 (6) | 16.5 ± 0.7 (10) | 0.84 ± 0.7 (4) |
| 7 weeks  | 161 ± 2.3 (15)  | 146 ± 2.9 (15) | 45.8 ± 2.7 (6) | 18.1 ± 0.6 (6) | 1.32 ± 0.1 (5) |
| 9 weeks  | 201 ± 2.7 (15)  | 189 ± 2.8 (15) | 43.0 ± 2.8 (6) | 16.3 ± 1.2 (8) | 1.76 ± 0.31 (5) |
| 15 weeks | 244 ± 3.4 (15)  | 228 ± 3.4 (15) | 45.7 ± 2.0 (6) | 19.6 ± 1.2 (8) | 1.97 ± 0.22 (5) |
| 20 weeks | 274 ± 11.0 (15)| 262 ± 7.9 (13) | 50.0 ± 1.9 (6) | 22.5 ± 1.6 (9) | 1.68 ± 0.05 (3) |

|          | P<0.001    | P<0.01     | P<0.001 | P<0.025 | P<0.01 |
|----------|------------|------------|----------|---------|---------|

* P values for fed versus fasted rats.

a Dashes indicate no significant difference between fed and fasted values (P > 0.05).

![Fig. 6](http://www.jbc.org/)

**DISCUSSION**

Several groups have isolated the vitamin D-dependent intestinal CaBP from avian and mammalian intestines and have reported on the properties of these proteins (18, 24, 25, 27, 28).
Vitamin D-dependent Calcium-binding Protein in Rat Intestine

The present report provides an alternative method for purifying rat CaBP which is rapid and yields milligram quantities. Previous molecular weight measurements of purified rat, bovine, and pig CaBP (18, 24, 27) compare favorably with our estimate of 10,000 molecular weight as assessed by gel filtration methodology. The vitamin D-dependent CaBP of birds is larger than the mammalian form, 28,000 molecular weight in a sodium dodecyl sulfate-polyacrylamide system. Bredederman and Wasserman, studying the calcium binding properties of purified chick CaBP, observed a high affinity site (Kd = 0.50 µM) with a stoichiometry of 4 mol of Ca2+/mol of protein (28). In the present study of rat intestinal CaBP, we observed a high affinity site for Ca2+ binding estimated at 0.3 µM with a binding capacity of ~2 mol of Ca2+/mol of protein. Freund and Bronner's (8) estimation for the dissociation constant of calcium binding to rat intestinal CaBP under different buffer conditions was 1.4 to 1.9 µM. The protein's calcium binding is consistent with our observations (Fig. 3) as well as with the studies of pig and bovine CaBP (24, 25) which demonstrated that EDTA produces large changes in the anionic properties of intestinal CaBP.

The present study is the first one which characterizes the changes in the duodenal CaBP content of normal growing rats. A close relationship between the degree of calcium absorption and growth has been noted previously (29-32). Nikolaessen originally observed that calcium absorption decreased early in a rat's life between 2 and 3 months of age at a time when the growth rate had slowed and the skeleton had essentially matured (29). In this present report, there was no change in CaBP units per proximal intestinal segment between 4 weeks (80 g) and 9 weeks (200 g) of age during the rapid growth phase. However, by 15 (240 g) and 20 (270 g) weeks of age, when weight gain had adjusted to a low level, the total CaBP activity had dropped by 54% and 70%, respectively. The observed age-related decrease in CaBP was due entirely to a decrease in quantity of CaBP rather than a modification of calcium binding sites. The decrease of CaBP/g of body weight followed an exponential decay with age similar to the exponential decrease in growth rate. These data indicate that factors controlling vitamin D-dependent CaBP may possibly mediate the changes in calcium absorption which attend growth.

Acute fasting, at all ages studied, markedly reduced the CaBP activity in the soluble fraction of duodenal extracts. As observed on polyacrylamide gels, fasting resulted in a specific decrease in the amount of CaBP. Although a low calcium diet and growth has been noted previously (29-32). Nicolaessen observed that calcium absorption decreased early in a rat's life between 2 and 3 months of age at a time when the growth rate had slowed and the skeleton had essentially matured (29). In this present report, there was no change in CaBP units per proximal intestinal segment between 4 weeks (80 g) and 9 weeks (200 g) of age during the rapid growth phase. However, by 15 (240 g) and 20 (270 g) weeks of age, when weight gain had adjusted to a low level, the total CaBP activity had dropped by 54% and 70%, respectively. The observed age-related decrease in CaBP was due entirely to a decrease in quantity of CaBP rather than a modification of calcium binding sites. The decrease of CaBP/g of body weight followed an exponential decay with age similar to the exponential decrease in growth rate. These data indicate that factors controlling vitamin D-dependent CaBP may possibly mediate the changes in calcium absorption which attend growth.

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