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SKIN CALCIUM-BINDING PROTEIN : EFFECT OF VITAMIN D DEFICIENCY AND VITAMIN D TREATMENT

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SUMMARY : The amount of skin calcium-binding protein, evaluated using a sensitive radioimmunoassay and indirect immunofluorescence, was decreased in vitamin-D deficient rats and increased after one week vitamin D_3 or 1α-hydroxyvitamin D_3 treatment. In vitamin D replete and in vitamin D-deficient animals, skin calcium-binding protein was not sensitive to changes in dietary and/or serum calcium concentrations. These results indicate that this protein is different from other calcium-binding proteins such as parvalbumin and calmodulin which are not vitamin D-dependent, and also different from intestinal calcium-binding protein which, in D replete animals, is sensitive to changes in dietary and serum calcium concentrations. Skin calcium-binding protein may, therefore, represent a new class of vitamin D-dependent protein.

INTRODUCTION : The synthesis of calcium-binding protein (CaBP) is firmly established as one of the molecular expressions of vitamin D action on intestinal cells (1). We have recently described a protein with calcium binding activity that is present in rat skin (SCaBP) (2, 3, 4). This protein shares many of the properties of other CaBPs : low molecular weight, cytoplasmic localization, acidic nature and high affinity for Ca. However, no immunological cross reactivity was observed with either calmodulin, S100, or intestinal CaBP (2, 5). Further characterization of SCaBP revealed many properties similar to parvalbumin (4) a muscle CaBP, which has not been established as being vitamin D-dependent (6, 7). To date, only two classes of calcium binding proteins have been described : those of MW 28,000, which are present in many avian and mammalian tissues (1) and those of MW 10,000 which are present in rat intestine (8, 9). It is therefore important to carefully examine the effect of vitamin D on the levels of SCaBP to determine if it represents a third class of vitamin D-dependent CaPBs, or if it is more like parvalbumin and calmodulin and, as such, is vitamin-D independent.

METHODS : Male wistar rats, 21 days old (Charles River, France) weighing about 48-50 g were housed in plastic cages (8 rats/cage). Food and water were allowed

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ad-libitum. In order to avoid vitamin D synthesis and diurnal changes in skin metabolism, the rats were housed in a dark room. The animals were raised on one of the following synthetic vitamin D-deficient diets : 0.45 % Ca, 2 % Ca or 0.02 % Ca. The appropriate amounts of other vitamins were given twice a week.

In the first series of experiments, one group of animals was raised on vitamin D-deficient 0.45 % Ca diet, and three others on diets containing 0.45 %, 0.02 % and 2.0 % Ca, but supplemented with 750 ng vitamin D_3 orally per day (crystalline cholecalciferol. Laboratoire Roussel, Paris, France) for a five week period. In a seperate experiment, rats were raised on 0.45 % Ca diet for four weeks, then divided into three groups, and given 750 ng of vitamin D_3 (in 0.1 ml peanut oil), 60 ng $1\alpha(OH)D_3$ (in 0.1 ml peanut oil. Laboratoire Léo, France) or solvent peanut oil, orally for 8 days. During this period, the pairfed method of feeding was applied. In the last experiment, rats were fed the 0.45 % Ca diet without vitamin D for four weeks, then the animals were divided in three groups, and given for one week before sacrifice one of the following regimen : 0.45 % Ca, 2 % Ca or 0.02 % Ca.

At the end of the experimental period, blood was collected form each animal, and serum calcium concentrations were determined. After removal of hair, skin samples (3 x 3 cm) from the dorsal region of each animal were taken, frozen in liquid nitrogen, pulverized in Spex-Mill (Spex metuche N.Y., U.S.A.) at liquid nitrogen temperature, and soluble proteins were obtained by centrifugation at 100,000 x g. Protein was measured by the method of Lowry (10), and SCaBP by a radioimmunoassay. For the assay, 4 µg of SCaBP, purified by the procedure previously described (2, 3) was iodinated [^{125}I] by the chloramine T method (11). Antiserum to highly purified SCaBP was generated in rabbits by standard techniques (2). The antiserum was tested using Ouchterlony double immunodiffusion technique and antibody precipitation technique (2, 5).

For the RIA, each tube contained sample or standard (0.1 ml in phosphate buffer, PB, 0.02 M, pH 7.4 + 0.02 % sodium azide containing 0.5 % of bovine serum albumin), diluted antibody (0.1 ml, working dilution from 1/2,500 to 1/10,000 in PB + BSA) and labeled SCaBP (0.1 ml, 10-12,000 cpm). After 16 h at 4°C, 0.1 ml of 0.5 % bovine γ globulin and 0.5 ml of 20 % polyethylenglycol 6,000 were added in order to separate free antigen from antigen-antibody complex.

After centrifugation, the radioactivity of the precipitated antigen-antibody complex was determined in Kontron MR480 automatic gamma counting system (Franco-Helvétique). The percent of maximum binding was calculated after correction for nonspecific binding (3.5 %). The SCaBP content of skin samples was calculted from a standard curve in which labeled protein was displaced from the antibody by increasing amounts of SCaBP in the range of 2.5-100 ng. The sensitivity of the assay was such that 2.5 ng of SCaBP could be measured (Fig.1).

Indirect immunofluorescence was performed as previously described (3).

RESULTS : When weaning rats were raised for 5 weeks on a vitamin D deficient-normal Ca (0.45 %) diet, SCaBP was still present in the cytosolic fraction of their skin but its amount was significantly ($P<0.001$) lower as compared to vitamin D-replete controls (Table 1). The effect of vitamin D status on SCaBP level was further confirmed by indirect immunofluorescence studies such as those shown in Fig.2. In vitamin D replete rats the immunofluorescence was found in the basal cell layer of the epidermis as previously described in normal rats (3). In vitamin D-deficient rats the distribution of the immuno-

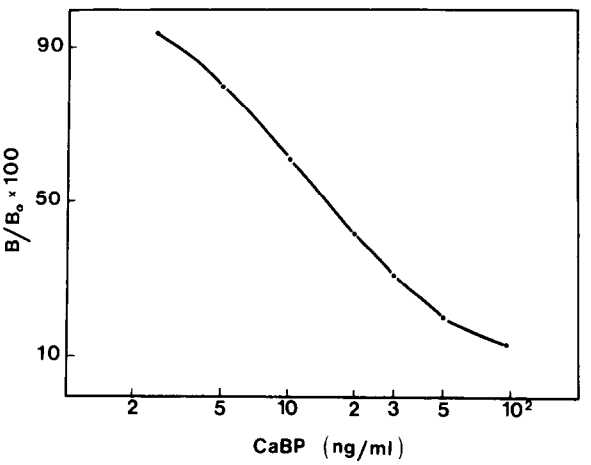

Figure 1 : Dose response curve for pure rat skin CaBP : Inhibition of [^{125}I] CaBP binding to anti-CaBP serum in the presence of increasing amounts of unlabeled CaBP : 2.5, 5, 10, 20, 30, 50 and 100 ng/ml.
B and B_0 are the precipitated radioactivity in the presence and absence of unlabeled CaBP respectively.

fluorescence was similar, but the intensity of the staining was decreased and some basal cells appeared unstained.

Vitamin D deficiency also resulted in a marked reduction in body weight and in severe hypocalcemia. In order to differentiate the specific action of vitamin D on SCaBP from that of the nutritional state of the animals on their protein synthesis, in the second experiment when D-deficient rats were treated for one week

Table 1 : Effects of vitamin D-deficiency (-Vit D) and of the daily administration of 750 ng of vitamin D_3 (+ Vit D) for 5 weeks on the amount of skin calcium binding protein (SCaBP), the body weight, and serum calcium concentration of weaning rats given a 0.45 % calcium diet.

	- Vit D	+ Vit D	
SCaBP (µg/mg cytosolic protein)	26.3 ± 2.7 (9)	47.8 ± 1.3 (6)	P<0.001
Serum calcium (mg/100 ml)	5.8 ± 0.14 (8)	10.7 ± 0.16 (8)	P<0.001
Body weight (g)	240.7 ± 6.4 (7)	261.7 ± 2.1 (6)	P<0.02

Number in parenthesis indicates number of rats.

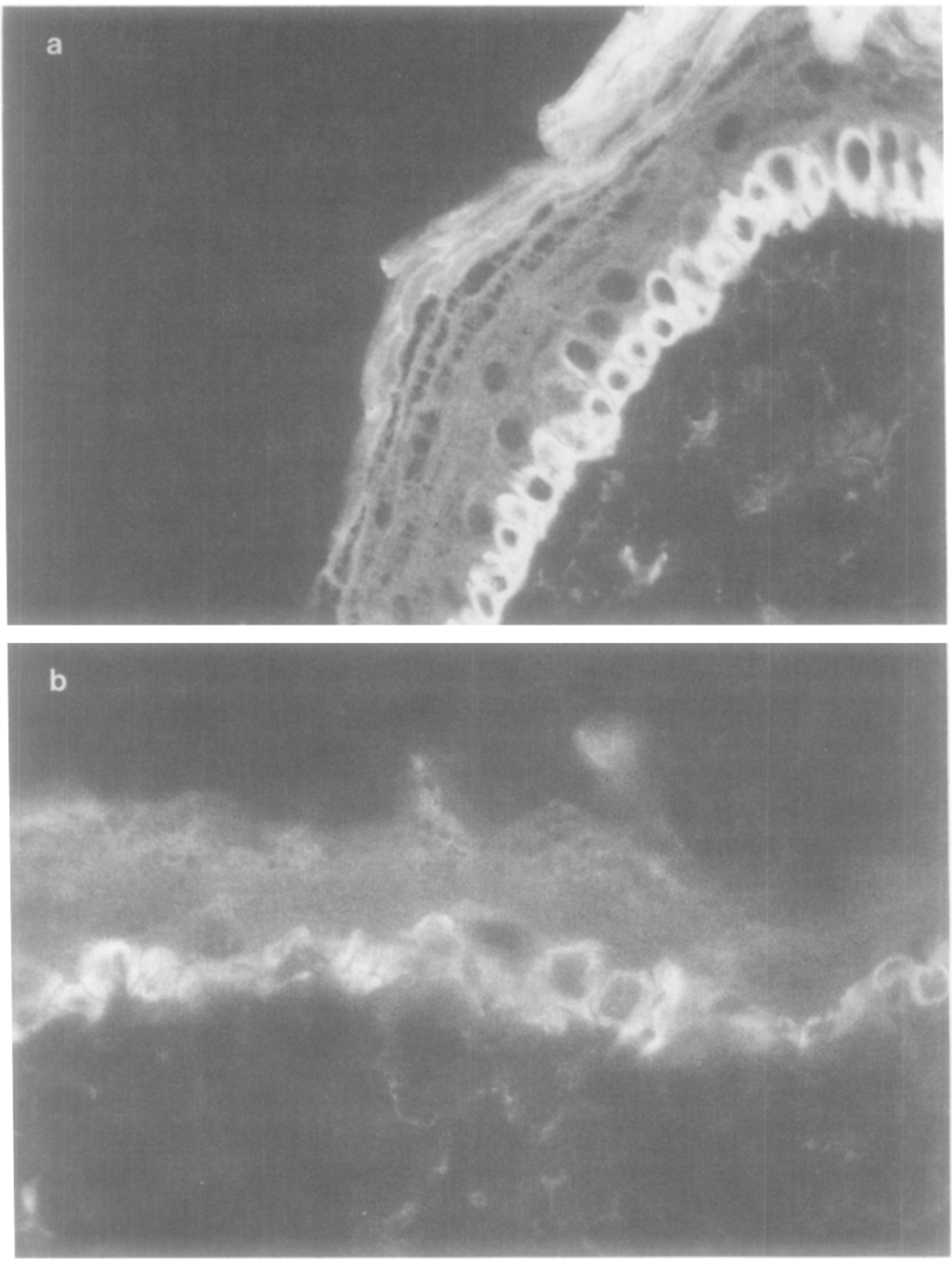

Figure 2 : Immunofluorescence with anti-SCaBP antisera on rat back skin frozen specimens.

- 2 (a) : Pattern of vitamin D repleted rats fed a 0.45 % calcium diet. Note the bright cytoplasmic staining of the basal cell in the epidermis. The fluorescence of the stratum corneum corresponds to a non specific staining as shown previously (3, 5). Antiserum diluted 1/20 (x 400).

- 2 (b) : Pattern of vitamin D deficient rats. The immunoreactivity is still localized in the basal cells but the staining is decreased. Anti-serum diluted 1/20 (x 400).

Table 2 : Effects of 8 days vitamin D_3 (750 ng/d) and 1α-hydroxyvitamin D_3 (60 ng/d) treatments on the amounts of skin calcium binding protein (SCaBP), serum calcium concentration, and body weight in pair-fed rats raised for 4 weeks on a D-deficient 0.45 % calcium diet. Controls were maintained on the D-deficient diet (- Vit D).

	- Vit D controls	+ Vit D	+ 1α-hydroxyvitamin D_3
SCaBP (μg/mg cytosolic protein)	20.5 ± 1.2 (5)	34.0 ± 4.8 * (5)	37.4 ± 6.0 * (6)
Serum calcium (mg/100 ml)	5.3 ± 0.2 (5)	9.6 ± 0.1** (6)	10.3 ± 0.2** (6)
Body weight (g)	246.6 ± 6.2 (5)	245.2 ± 6.2 (5)	255.4 ± 3.6 (6)

*P<0.05 compared to - Vit D ; **P<0.001 compared to - Vit D.

with either vitamin D_3, 1α(OH)D_3 or the vehicle, animals were pair-fed. Under these experimental conditions body weights were comparable in all three groups of rats (Table 2). However, a significant increase in SCaBP was observed in vitamin D_3 or 1α(OH)D_3 treated rats. As anticipated serum calcium was normalized by such treatments.

Similarly, it was important to determine whether changes in serum calcium concentration could modify the amount of SCaBP. For this purpose diets containing either 0.02 % Ca or 2 % Ca, were given for 5 weeks to rats treated daily with 750 ng of vitamin D, also these two diets, were given in one week to vitamin D-deficient rats previously raised on a 0.45 % calcium diet. Controls in both experiments received the normal 0.45 % calcium diet. As shown on table 3, in spite of significantly different serum calcium concentrations and severe hypocalcemia in 0.02 % Ca fed animals, SCaBP was comparable in the three groups of D replete rats. In D-deficient animals SCaBP was low and the levels comparable in the three groups receiving different Ca regimen, although the poor calcium diet provoked a further decrease in serum calcium concentration and the Ca-rich diet induced a significant rise in serum calcium (Table 4).

DISCUSSION : Vitamin D through its active metabolite, 1,25-dihydroxyvitamin D_3 acts on its target tissues to increase protein synthesis (12, 13). One protein that has been extensively studied is the in-

Table 3 : Effects of 5 weeks low-calcium (0.02 %) or high-calcium (2 %) diet in vitamin D replete rats (750 ng/d vitamin D_3) on their SCaBP, serum calcium concentration, and body weight. Controls were raised on the 0.45 % calcium diet.

Dietary calcium	Controls (0.45 %)	Low-Ca (0.02 %)	High-Ca (2 %)
SCaBP (µg/mg cytosolic protein)	47.8 ± 1.3 (6)	51.4 ± 3.2 (5)	50.5 ± 2.3 (8)
Serum calcium (mg/100 ml)	10.7 ± 0.2 (8)	5.0 ± 0.2 * (7)	11.1 ± 0.1 (8)
Body weight (g)	261.7 ± 2.2 (6)	147.8 ± 2 4 * (6)	270.0 ± 4.8 (7)

* P<0.001 compared to 0.45 % calcium diet.

testinal calcium binding protein. The mRNA and the levels of this protein are increased after $1,25(OH)_2D_3$ administration (14, 15). Skin also contains a calcium binding protein, but this protein is distinct from the one found in the intestine of both mammals and birds. It resembles more closely parvalbumin, a muscle CaBP, in terms of its amino acid composition and calcium binding properties (4). It was, therefore, important to determine if SCaBP is like intestinal

Table 4 : Effects of one week low-calcium (0.02 %) or high-calcium (2 %) diet in vitamin D-deficient rats on their SCaBP, serum calcium concentration, and body weight. Controls were maintained on the previous 0.45 % calcium-Vitamin D deficient diet.

Dietary calcium	Controls (0.45 %)	Low-Ca (0.02 %)	High-Ca (2 %)
SCaBP (µg/mg cytosolic protein)	28.4 ± 2.2 (6)	34.3 ± 2.4 (8)	27.8 ± 1.6 (7)
Serum calcium (mg/ml)	5.1 ± 0.3 (6)	4.6 ± 0.1 (8)	8.5 ± 0.3** (5)
Body weight (g)	190.7 ± 6.2 (6)	173.8 ± 5.4 (8)	189.1 ± 7.4 (8)

Compared to controls : **P<0.001. Number in parenthesis indicates number of rats.

CaBP in its vitamin D-dependence or like parvalbumin in its vitamin D independence.

The results of this study clearly show the vitamin D-dependence of SCaBP. Rats deprived of vitamin D for 5 weeks after weaning have significantly decreased SCaBP and the amount of this protein increases to control values after the administration of vitamin D_3 or $1\alpha(OH)D_3$ for one week to D-deficient animals. Thus, SCaBP seems to be different from parvalbumin and more like intestinal CaBP. However, there are subtle differences between SCaBP and intestinal CaBP. Vitamin D deficiency leads to a decrease in intestinal CaBP to very low levels whereas the decrease in SCaBP is less important. Approximately 50 % of the basal level are still present in the D-deficient animal, i.e., 26.3 $\pm$ 2.7 *vs* 47.8 $\pm$ 1.3 µg/mg cytosolic protein. In addition, it has been demonstrated that an increase in intestinal CaBP occurs after administration of a low Ca diet, and a decrease follows the administration of a Ca rich diet (16-18). Our data from D-replete rats as well as from D-deficient animals indicate that SCaBP is not sensitive to changes in dietary calcium nor in extracellular calcium concentrations.

We have recently shown that the increase in SCaBP after vitamin D treatment is associated, in the rat, with changes in epidermopoiesis (19). Moreover, in human studies we reported that SCaBP, which is normally absent in the columnar epithelium, will appear "de novo" when this tissue assumes the characteristics of squamous epithelium, i.e., squamous dysplasia (20). These observations put together suggest that SCaBP may represent a new class of vitamin D-dependent protein more closely related to cellular growth and differentiation than to the presence of vitamin D or its active derivative "per se". In this view, the effect of vitamin D on SCaBP could be explained through the action of its active metabolite $1\alpha,25(OH)_2D_3$ on the terminal differentiation of epidermal cells, as shown in mouse epidermal cells in primary culture (21).

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