INTESTINAL CALMODULIN AND CALCIUM-BINDING PROTEIN DIFFER IN THEIR DISTRIBUTION AND IN THE EFFECT OF VITAMIN D STEROIDS ON THEIR CONCENTRATION

Monique THOMASSET, Annie MOLLA*, Owen PARKES and Jacques G. DEMAILLE*,+

Unité de Recherches sur le Métabolisme Hydro-Minéral, INSERM U-120 (Dr Pr H Mathieu), 44 Chemin de Ronde, 78110 Le Vésinet and **Centre de Recherches de Biochimie Macromoléculaire du CNRS, Unité INSERM Biochimie des Régulations and Faculté de Médecine, BP 5015, 34033-Montpellier, France*

Received 7 March 1981

1. Introduction

While the absorption of calcium by the intestine is a major component of the body overall calcium homeostatic system, the mechanism of the process itself remains poorly understood. One of the most important cytoplasmic calcium receptors is calmodulin, an ubiquitous calcium-binding protein that regulates the activity of several calcium-dependent enzymes, including cyclic nucleotide phosphodiesterase, adenylate cyclase, Ca^{2+},Mg^{2+}-ATPase, and myosin light-chain kinase (reviews [1–3]). Calmodulin has been located in brush borders isolated from intestinal epithelial cells in the rat [4]. Although some studies support the hypothesis that calmodulin is involved in microvillus motility, its exact function in the intestinal brush border is not yet established [4,5]. There is evidence indicating that calmodulin acts as a modulator for the transmembrane transport of calcium in sarcoplasmic reticulum [6] and the erythrocyte membrane [7]. To date, however, there has been no report of its involvement in intestinal calcium transport.

Another calcium-binding protein (CaBP), vitamin D-dependent CaBP, is also found in the small intestine, and its appearance has been associated with vitamin D-dependent calcium absorption [8,9]. Like calmodulin, CaBP is a small (11 000 M_r), acidic protein, and seems to be associated with the activation of at least one enzyme [10]. The calcium-binding constants for the 2 proteins are remarkably similar ($K_a \simeq 10^6$ M) and they share a number of other physicochemical features [11]. They are indeed derived from a common evolutionary ancestor [12]. However, vitamin D-dependent CaBP is not restricted to the brush border of the enterocyte, [13] but is distributed throughout the cell with a reported concentration in the terminal web region [14]. As the 2 calcium-binding proteins have been located in the gut we undertook this study of the distribution of calmodulin and CaBP along the length of the rat small intestine. Furthermore, because the vitamin D status is of great importance in the regulation of intestinal calcium absorption [15], the concentration of the proteins was also determined under varying conditions of vitamin D deficiency and repletion. The acute response to changes in vitamin D-steroid level was followed after administration of $1,25(OH)_2D_3$, the hormonal form of vitamin D [15].

2. Materials and methods

2.1. *Animals and diets*

Sprague-Dawley rats (Charles River, France) were used throughout the experiments. They were raised in the dark and had free access to deionized water. Vitamin D-deficient rats (–D) were obtained from females fed a vitamin D-free diet from weaning for 6 months. Diets contained 0.50% calcium, 0.36% phosphorus except for the gestation and lactation periods when both calcium and phosphorus levels were increased to 0.90%. After weaning the –D rats were fed a vitamin D-free diet containing 0.50% calcium, 0.36% phos-

Abbreviations: CaBP, calcium binding protein; $1,25(OH)_2D_3$, 1,25 dihydroxy vitamin D_3

+ To whom correspondence and reprint requests should be addressed

phorus. Half of the –D animals were injected intraperitoneally with $1,25(OH)_2D_3$ in 10 μl 95% ethanol (100 ng/100 g body wt) 48 h and 24 h before sacrifice (–D + $1,25(OH)_2D_3$). Vitamin D-supplemented rats (+D) were obtained by the same protocol, but mothers and the small rats after weaning received vitamin D_3-supplemented diets (4000 and 2000 IU/kg diet, respectively). All the animals in the 3 groups (–D, –D + $1,25(OH)_2D_3$, +D) were sacrificed by exsanguination 9 days after weaning. Blood was collected and the serum analyzed for calcium by atomic absorption spectrophotometry.

2.2. *Preparation of tissue*

The whole duodenum from the pyloric sphincter to the angle of Treitz (5 cm), 8 cm of mid-jejunum and 8 cm of distal ileum from each animal were excised and everted. The mucosal tissue, obtained by scraping with a glass slide at 4°C, was divided into 2 for CaBP and calmodulin analyses, respectively.

2.3. *CaBP determinations*

The mucosal tissue was homogenized in 4 vol. Tris buffer (0.12 M NaCl, 3 mM KCl, 13 mM Tris–HCl (pH 7.4)) centrifuged at 100 000 × *g* for 1 h at 4°C and the supernatant fraction stored at –30°C. CaBP was quantified directly by radioimmunoassay according to [16], using a final antiserum dilution of 1/60 000. The CaBP content was expressed as μmol/kg fresh mucosa assuming for CaBP, M_r = 10 000 [17].

2.4. *Calmodulin determinations*

The lyophilized mucosal tissue was homogenized in 3 ml buffer (1 mM Mg-acetate, 20 μM $CaCl_2$, 10 mM Tris–HCl (pH 7.5)) and centrifuged at 24 000 × *g* for 5 min at 4°C. The resulting supernatant was heated at 60°C for 5 min, cooled to 4°C and centrifuged at 24 000 × *g* for 10 min. The final supernatant was stored at –30°C for calmodulin determination. Calmodulin was assayed by the activation of calcium–calmodulin-dependent myosin light chain kinase, following the procedure in [18], as modified [19]. The final dilution of the extract in the assay was 320-fold and was found sufficient to obtain the maximal specific activity. Apparent K_d of the kinase for calmodulin was 32 nM. Calmodulin concentration was expressed as μmol/kg fresh mucosa.

3. Results

The average plasma calcium level of the control, vitamin D_3-supplemented rats was found to be 10.8 ± 0.1 mg/dl and that for the $1,25(OH)_2D_3$-dosed animals was similar at 11.2 ± 0.2 mg/dl. The plasma calcium levels of the vitamin D-deficient rats were, however, considerably lower at 6.7 ± 0.3 mg/dl.

The distribution of immunoreactive CaBP along the intestine is presented in table 1. The major concentration is in the duodenum, which contains up to 40-times more protein than the other 2 regions. The

Table 1
Calcium-binding protein (CaBP) concentrations (μmol/kg fresh mucosa) in duodenum, jejunum and ileum

Diet	Duodenum (Dd)	Jejunum (Jj)	Ileum (Il)	Statistical analysis Dd *vs* Jj and Dd *vs* Il	Jj *vs* Il
–D	16.6 ± 3.2 (7)	0.6 ± 0.10 (4)	0.1 ± 0.02 (4)	$P < 0.001$	$P < 0.01$
–D + $1,25(OH)_2D_3$	89.0 ± 15.7 (6)	3.1 ± 0.4 (3)	4.6 ± 1.1 (3)	$P < 0.001$	n.s.
+D	62.4 ± 14.9 (8)	2.0 ± 0.5 (4)	5.1 ± 1.9 (4)	$P < 0.001$	n.s.
Statistical analysis					
–D *vs* + D	$P < 0.02$	$P < 0.05$	$P < 0.05$		
–D *vs* –D + $1,25(OH)_2D_3$	$P < 0.02$	$P < 0.01$	$P < 0.02$		

The results are the mean ± SEM. The figures in parentheses represent the number of rats. Analysis for statistical significance was performed by Student's *t*-test; n.s., not significant

Table 2
Calmodulin concentrations (μmol/kg fresh mucosa) in duodenum, jejunum and ileum

Diet	Duodenum (Dd)	Jejunum (Jj)	Ileum (Il)	Statistical analysis (Dd/Jj)	(Dd/Il)
−D	101 ± 4.7 (7)	137 ± 14.0 (3)	80 ± 5.9 (3)	n.s.	$P < 0.05$
−D + $1,25(OH)_2D_3$	90 ± 6.8 (5)	122 ± 12.4 (3)	91 ± 12.0 (3)	$P < 0.01$	n.s.
+D	120 ± 7.7 (8)	112 ± 21.9 (4)	87 ± 3.7 (4)	n.s.	$P < 0.02$
Statistical analysis					
−D *vs* + D	n.s.	n.s.	n.s.		
−D *vs* −D + $1,25(OH)_2D_3$	n.s.	n.s.	n.s.		

The results are the mean ± SEM. The figures in parentheses represent the number of rats. Analysis for statistical significance was performed by Student's *t*-test; n.s., not significant

CaBP content of all 3 regions of small intestine is not significantly different between animals raised on a vitamin D_3-supplemented diet and those given an acute $1,25(OH)_2D_3$ treatment. In vitamin D-deficient animals the ileal and jejunal concentrations dropped close to zero, but the duodenum contained a reduced but still significant quantity of the protein (~20% of the vitamin D-replete value).

Calmodulin, as shown in table 2, is found in even greater molar concentration than CaBP. The levels in all 3 sections of the small intestine are similar at ~100 μmol/kg mucosa. The only area with a slightly reduced concentration is the ileum. The concentration of calmodulin under all 3 of the dietary conditions studied (−D, +D, −D + $1,25(OH)_2D_3$) remained unchanged, both in the duodenum and in the other 2 regions of the small intestine.

4. Discussion

Whereas [4,5] established the presence of calmodulin in the intestinal epithelium, this is the first time that the protein has been quantitatively studied in this tissue. The concentration of calmodulin throughout the length of the small intestine is remarkably high. Such values could only be measured at high sample dilutions, up to constant specific activity [19]. They appear to be even greater than those of amphibian oocytes exposed to progesterone (44–59 μmol/kg in [19]) or of brain and smooth muscle, measured by Coomassie blue binding (40 and 24 μmol/kg, respectively [20]). This is in line with the finding that calmodulin is one of the major proteins of the microvillus core [4].

The level of the other calcium-binding protein, CaBP, is similar in the duodenum of $1,25(OH)_2D_3$-treated rats, so that, between them, these 2 proteins account for ~5% of the soluble protein in duodenal mucosa. The concentrations of the 2 proteins are only similar in the duodenum, however. In the other 2 regions of the small intestine there is little change in calmodulin while CaBP is reduced to 2% of its duodenal level. The differential distribution of CaBP correlates quite readily with the reported active transport of calcium, which is also highest in the duodenum [21]. However, both the ileum and jejunum are involved in calcium absorption and in them the level of calmodulin remains high.

When tritiated-$1,25(OH)_2D_3$ is given to vitamin D-deficient rats it was found that the steroid is most concentrated in the duodenum [22] with lesser amounts in the other 2 regions of the small intestine. Our measurements of CaBP levels correlate very closely with this observation in that the maximum vitamin D-dependent synthesis of CaBP occurs in the duodenum. Nevertheless, the ileal and jejunal levels of CaBP are influenced, suggesting that vitamin D-dependent calcium absorption is not confined to the duodenum. Neither is CaBP the only protein which is vitamin D-dependent. A $1,25(OH)_2D_3$-stimulated actin-like protein of the brush border was found [23] and the activities of 2 enzymes, alkaline phosphatase and Ca^{2+}-dependent ATPase are also vitamin D-dependent [24].

The interrelationship of these proteins remains to be defined.

The intestinal level of calmodulin, on the other hand, appears to be quite independent of vitamin D. A similar lack of sensitivity of the erythrocyte calmodulin concentration to the vitamin D status was reported [25].

The fact that calmodulin is almost equally distributed in all portions of the small intestine and that its concentration is not modified upon vitamin D withdrawal or repletion argues in favor of different physiological roles for CaBP and calmodulin. The latter was recently proposed to serve as a calcium buffer modulating the free calcium concentration of microvilli [26]. Also, calmodulin-dependent myosin light chain kinase was found to be present in intestinal epithelial cells (Le Peuch, D. A. M., Le Peuch, C. J. and J. G. D., unpublished) where it is presumably involved in the control of the interaction of actin and myosin, the latter being mostly present in the terminal web [27]. Calmodulin may also be involved in the regulation of Ca^{2+} fluxes through membranes, even though there is as yet no evidence for such regulation.

Acknowledgements

We are grateful to N. Gouhier and J. Saliba for expert technical assistance. We are indebted to Dr Moukhtar (INSERM U-113, Paris) for allowing us to use his laboratory to prepare ^{125}I-labeled CaBP and to Dr M. Uskokovic (Hoffmann-La Roche, Nutley NJ) for supplying $1,25(OH)_2D_3$. This investigation was supported in part by a Xavier Bichat grant to M. T. and by grants to J. G. D. from CNRS (Mécanisme d'action des hormones au niveau cellulaire), INSERM (ATP 63.78.95 Fibre musculaire cardiaque, CRL 78.4.086.1, 79.4.151.3 and 80.5.032), DGRST (ACC BFM and Parois artérielles), Muscular Dystrophy Association, Inc., Fondation pour la Recherche médicale and NATO grant no. 1688. A. M. acknowledges receipt of a DGRST fellowship.

References

[1] Klee, C. B., Crouch, T. H. and Richman, P. G. (1980) Ann. Rev. Biochem. 49, 489–515.
[2] Cheung, W. Y. (1980) Science 207, 19–27.
[3] Means, A. R. and Dedman, J. R. (1980) Nature 285, 73–77.
[4] Howe, C. L., Mooseker, M. S. and Graves, T. A. (1980) J. Cell. Biol. 85, 916–923.
[5] Glenney, J. R. and Weber, K. (1980) J. Biol. Chem. 255, 10551–10554.
[6] Le Peuch, C. J., Haiech, J. and Demaille, J. G. (1979) Biochemistry 18, 5150–5157.
[7] Larsen, F. L. and Vincenzi, F. F. (1979) Science 204, 306–309.
[8] Wasserman, R. H. and Taylor, A. N. (1968) J. Biol. Chem. 243, 3987–3993.
[9] Thomasset, M., Cuisinier-Gleizes, P. and Mathieu, H. (1979) FEBS Lett. 107, 91–94.
[10] Freund, T. S. (1980) in: Calcium Binding Proteins: Structure and Function (Siegel, F. L. et al. eds) p. 407, Elsevier/North-Holland, Amsterdam, New York.
[11] Wasserman, R. H. (1980) in: Calcium Binding Proteins: Structure and Function (Siegel, F. L. et al. eds) p. 357, Elsevier/North-Holland, Amsterdam, New York.
[12] Demaille, J G., Haiech, J. and Goodman, M. (1980) Prot. Biol. Fluids 28, 95–98.
[13] Jande, S. S., Tolnai, S. and Lawson, D. E. (1980) in: Calcium Binding Proteins: Structure and Function (Siegel, F. L. et al. eds) p. 409, Elsevier/North-Holland, Amsterdam, New York.
[14] Marche, P., Cassier, P. and Mathieu, H. (1980) Cell Tiss. Res. 212, 63–72.
[15] Omdahl, J. L. and DeLuca, H. F. (1973) Physiol. Rev. 53, 327–371.
[16] Marche, P., Pradelles, P., Gros, C. and Thomasset, M. (1977) Biochem. Biophys. Res. Commun. 76, 1020–1025.
[17] Freund, T. S. and Bronner, F. (1975) Am. J. Physiol. 228, 861–869.
[18] Le Peuch, C. J., Ferraz, C., Walsh, M. P., Demaille, J. G. and Fischer, E. H. (1979) Biochemistry 18, 5267–5273.
[19] Cartaud, A., Ozon, R., Walsh, M. P., Haiech, J. and Demaille, J G. (1980) J. Biol. Chem. 255, 9404–9408.
[20] Grand, R. J. A. and Perry, S. V. (1979) Biochem. J. 183, 285–295.
[21] Krawitt, E. L. and Schedl, H. P. (1968) Am. J. Physiol. 214, 232–236.
[22] Stumpf, W. E., Sar, M., Reid, F. A., Tanaka, Y. and DeLuca, H. F. (1979) Science 206, 1188–1190.
[23] Wilson, P. W. and Lawson, D. E. M. (1978) Biochem. J. 173, 627–631.
[24] Haussler, M. R., Nagode, L. A. and Rasmussen, H. (1970) Nature 228, 1199–1201.
[25] Halloran, B. P., DeLuca, H. F. and Vincenzi, F. F. (1980) FEBS Lett. 114, 89–92.
[26] Glenney, J. R. jr, Bretscher, A. and Weber, K. (1980) Proc. Natl. Acad. Sci USA 77, 6458–6462.
[27] Drenckhahn, D. and Groschel-Stewart, U. (1980) J. Cell Biol. 86, 475–482.