Journal of Steroid Biochemistry, 1978, Vol. 9, pp. 159–162. Pergamon Press. Printed in Great Britain

DUODENAL CALCIUM-BINDING PROTEIN (CaBP) AND BONE CALCIUM MOBILIZATION IN RESPONSE TO 24R,25 AND 24S,25-DIHYDROXYCHOLECALCIFEROL IN INTACT AND NEPHRECTOMIZED RATS

M. THOMASSET*, J. REDEL†, P. MARCHE and P. CUISINIER-GLEIZES
Unité de Recherches sur le Métabolisme Hydro-Minéral, INSERM U.120, 44 Chemin de Ronde, 78110 Le Vesinet and
†Institut de Rhumatologie, Centre de Recherches sur les Maladies Ostéo-Articulaires (INSERM U.5, ERA 337 CNRS) Hôpital Cochin, 27 rue du Fg St Jacques, 75674 Paris Cédex 14, France

(*Received* 9 *June* 1977)

SUMMARY

As the duodenal calcium-binding protein (CaBP) can be considered as the molecular expression of the effect of cholecalciferol on the intestine, we investigated the respective effect of 24R,25 and 24S,25-$(OH)_2D_3$ on duodenal CaBP production in vitamin D and calcium-deficient rats. Our results showed a linear relationship between CaBP synthesis and the logarithm of the dose of either 24R or 24S epimers. The dose-response curves were parallel and displayed that the 24R epimer was about twice as active as the 24S epimer to promote CaBP synthesis. Similarly bone calcium mobilization response was dose related as a linear function of the logarithm of the administered dose. Again the 24R epimer was about twice as effective as the 24S epimer. However 24R and 24S epimers were respectively four and eight times less active than 1α,25-$(OH)_2D_3$ in stimulating bone response. Contrary to the CaBP response, the bone response was maximal and saturable from doses of 1040 pmol of 24R or 24S epimers. Both stereoisomers were more effective in promoting CaBP synthesis than in stimulating bone calcium mobilization. Furthermore bilateral nephrectomy prevents duodenal CaBP response to a large dose (1040 pmol) of 24R,25-$(OH)_2D_3$.

INTRODUCTION

Vitamin D_3 or cholecalciferol is first hydroxylated on carbon 25 to produce 25 hydroxycholecalciferol (25-OHD_3) before it is further metabolized to either 1α,25-dihydroxycholecalciferol (1α,25-$(OH)_2D_3$) or to 24,25-dihydroxycholecalciferol (24,25-$(OH)_2D_3$). 1α,25-$(OH)_2D_3$ appears the most active metabolite that is regarded as a calcium-regulating hormone [7] whereas the function of 24,25-$(OH)_2D_3$ remains relatively unclear [2]. The configuration of the C-24 hydroxyl group may be 24R or 24S, although the 24R epimer is the only one formed *in vivo* [11]. In rats, the biological activity of synthetic 24R and 24S epimers was mostly investigated in the intestinal calcium transport and bone calcium mobilization [6, 11]. In these systems, their effectiveness seems subsequent of the 1α-hydroxylation in the kidney [11].

Since the synthesis of a soluble calcium-binding protein (CaBP) can be considered as a sensitive index of the intestinal response to cholecalciferol [1] the purpose of the present study is to investigate the respective effect of 24R,25 and 24S,25-$(OH)_2D_3$ on duodenal CaBP and show whether they have a discriminatory influence on CaBP and bone calcium mobilization or not. To this end log-dose response curves have been established in terms of CaBP synthesis or in terms of increase in serum calcium in vitamin D-deficient rats fed a low-calcium diet. Furthermore we have measured the duodenal CaBP and serum calcium responses to an effective dose of 24R,25-$(OH)_2D_3$ in nephrectomized rats.

24R,25 and 24S,25-$(OH)_2D_3$ used were chemically synthesized by one of us [8]. A preliminary report of a part of this work has been previously presented [12].

EXPERIMENTAL

Animals and diets. Male weanling rats (40–50 g) of the Sprague strain were fed *ad libitum* a vitamin D-deficient diet‡ containing (w/w) 0.5% calcium and 0.36% phosphorus during 4 weeks. Serum calcium was measured at weekly intervals. For one additional week, the hypocalcemic rats (< 6.5 mg Ca/100 ml of serum) considered as vitamin D-deficient animals were kept on a low-calcium vitamin D-deficient diet‡ containing (w/w) 0.03% calcium and 0.36% phosphorus. Deionized water was supplied *ad libitum*. Thereafter, each rat received a single intravenous injection of various doses of 24R,25 or 24S,25-$(OH)_2D_3$ in ethanol. In all experiments some vitamin D-deficient control animals received the appropriate volume

* To whom correspondence should be sent.

‡ The composition of the regimen was (in % w/w): D-glucose, 38; vitamin-free casein, 18; starch, 31; peanut oil, 8; barm, 3; salt mixture 3 or 2 according to the diet used; and 5.000 u. of vitamin A per kg of diet.

of vehicle. Forty-eight h later all rats were sacrificed by exsanguination. In the experiments on nephrectomized rats the animals were anesthetized with ether and subjected to either a sham operation or total bilateral nephrectomy through a single midline dorsal incision deflected over both flanks. Immediately after surgery the rats received an intravenous injection of either steroid or vehicle. Measurements were performed 24 h later, a time compatible with the survival period of nephrectomized rats.

Measurement—duodenal CaBP. The proximal 15 cm of intestine was removed, everted and the mucosal tissue was scraped. The pooled material from 6 animals was treated as previously described [5]. A low molecular weight fraction was isolated from the duodenal mucosa by Sephadex G75 chromatography. The CaBP content was obtained from the CaBP activity (maximum binding nmol Ca^{2+} bound/mg protein) quantitated by saturation analysis using a [^{45}Ca]-Chelex 100 assay according to the method of Freund and Bronner [5]. The data were analyzed by the method of Scatchard [9]. For purpose of quantitative comparisons, equal amounts (150 mg) of protein in supernate were loaded onto the column.

Protein was monitored at 280 nm. ^{45}Ca was analyzed by liquid scintillimetry.

Bone calcium mobilization. The increase in serum calcium after the injection of steroid in vitamin D-deficient rats fed a low-calcium diet is regarded as resulting from the mobilization of bone calcium. Serum calcium was determined by atomic absorption spectrophotometry. Analysis for statistical significance was performed by means of variance analysis and then by Student's *t*-test. From our dose–response curves, the relative effectiveness of the 24R epimer as compared to that of the 24S epimer was calculated and expressed as the coefficient of concentration ρ.

RESULTS

The time course of the calcemic response to 260 pmol of 24R,25-$(OH)_2D_3$ or 520 pmol of 24S,25-$(OH)_2D_3$ is shown in Fig. 1. When using these respective doses there was not a significant difference between the response to the R and S epimers at any time studied. On the other hand both epimers were unefficient 12 h following their administration but significantly active after 24 or 48 h ($P < 0.01$) without any noticeable difference between these time points. Note that the calcemic response to 65 pmol of 1α,25-$(OH)_2D_3$ was significant ($P < 0.01$) after 12, 24 and 48 h. The magnitude of the response after 12 and 24 h was higher ($P < 0.001$) than the one obtained with dose of 24R,25 and 24S,25-$(OH)_2D_3$ respectively four or eight times greater.

A detailed dose–response study was then carried out after 48 h, a time compatible to detect both maximal calcemic response and CaBP [5]. The CaBP response was dose related and can be described as a

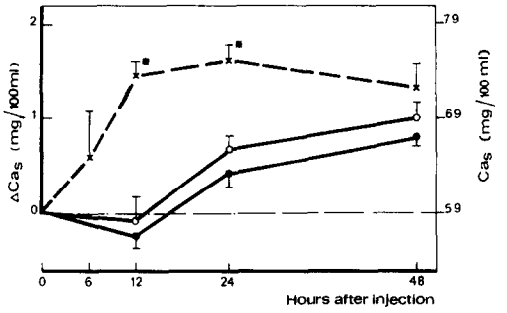

Fig. 1. Time course of the response of bone calcium mobilization to 65 pmol (0.025 μg) of 1α,25-$(OH)_2D_3$ (×) or 260 pmol (0.1 μg) of 24R,25-$(OH)_2D_3$ (●) or 520 pmol (0.4 μg) of 24S,25-$(OH)_2D_3$ (○). Serum calcium was measured before and at varying intervals following a single intravenous injection of steroid to vitamin D and calcium-deficient rats. Values are the mean ± S.E.M. of six rats. * 1α,25-$(OH)_2D_3$ significantly different from 24R,25 and 24S,25-$(OH)_2D_3$ $P < 0.01$.

linear function of the logarithm of the administered dose (Fig. 2). This response was considered as significant (values at least twice higher than the one of control) with 130 pmol of 24R,25-$(OH)_2D_3$ and 520 pmol of 24S,25-$(OH)_2D_3$. As with the CaBP response, the calcemic response was dose related and can be represented as a linear function of the logarithm of the dose (Fig. 3). Bone calcium mobilization was significantly ($P < 0.01$) stimulated by 260 pmol of 24R,25-$(OH)_2D_3$ and 520 pmol of 24S,25-$(OH)_2D_3$. Moreover there was not a significant difference in the rise of serum calcium produced at larger doses as 1040 and 2080 pmol of 24R,25 or 24S,25-$(OH)_2D_3$. In this range of doses the saturation of the effectiveness of these compounds in stimulating duodenal CaBP was only detectable for the S epimer (Fig. 2).

The linearity of the relationship between the increment of CaBP and the rise in serum calcium is displayed in Fig. 4. The two stereoisomers would thus appear to be more effective in promoting CaBP synthesis than bone calcium mobilization. Indeed 130 pmol of 24R epimer or 260 pmol of 24S epimer

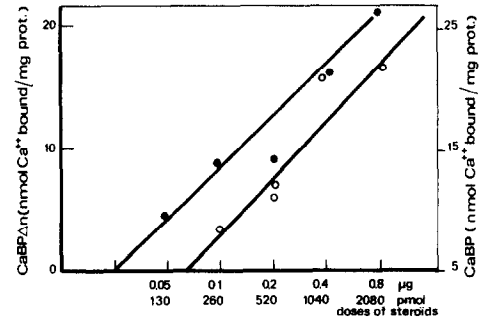

Fig. 2. Response of duodenal calcium-binding protein (CaBP) to increasing doses of 24R,25-(●) or 24S,25-$(OH)_2D_3$ (○) 48 h after intravenous steroid administration to vitamin D-deficient rats on a calcium-free diet. $\Delta n = n$ experimental animals − n control. The equations of the regression lines are: (●) $\Delta n = 0.04\ (\pm 0.870) + 13.7\ (\pm 2.34)$ log dose. $r = 0.96$ $p < 0.02$ (○) $\Delta n = -6.5\ (\pm 1.94) + 15.5\ (\pm 3.26)$ log dose. $r = 0.94$ $p < 0.02$ where dose is units (1 I.U. = 65 pmol) and the values in parentheses are S.D.

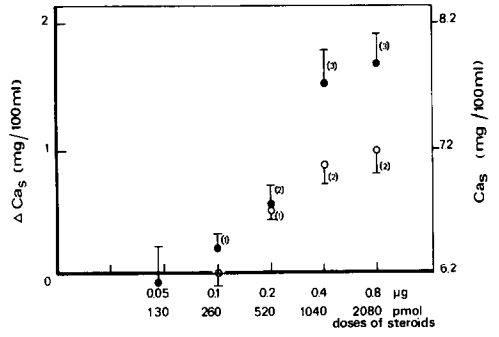

Fig. 3. Response of bone calcium mobilization to increasing doses of 24R,25-(●) or 24S,25-(OH)$_2$D$_3$ (○) 48 h after intravenous steroid administration to vitamin D-deficient rats fed a low calcium diet. $\Delta Ca_s = Ca_s$ 48 h $- Ca_2$ 0 h. There were 6 rats in each group. The vertical bars represent the S.E.M. of ΔCa_s values. Significance vs Ca_s at 0 h (1) $P < 0.05$ (2) $P < 0.01$ (3) $P < 0.001$. The equations of the regression lines are: (●) $\Delta Ca_s = -0.8$ ($\pm$ 0.06) + 1.9 ($\pm$ 0.29) log dose. $r = 0.96$ $P < 0.01$ (○) $\Delta Ca_s = -0.7$ ($\pm$ 0.13) + 1.2 ($\pm$ 0.36) log dose. $r = 0.95$ $P < 0.02$ where dose is units and the values in parentheses are S.D.

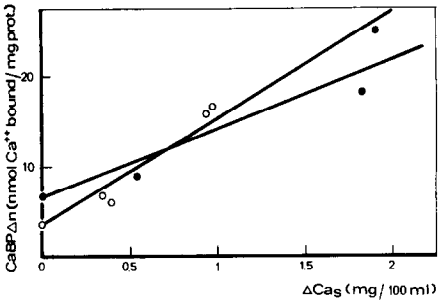

Fig. 4. Increment of duodenal calcium-binding protein levels, Δn: nmol Ca^{++} bound/mg protein as a function of increment in serum calcium (ΔCa_s: mg/100 ml) in vitamin D and calcium-deficient rats treated with increasing doses of 24R,25-(●) or 24S,25-dihydroxycholecalciferol (○) administered 40 hours earlier. The equations of the regression lines are: (●) $\Delta n = 6.2$ ($\pm$ 2.05) + 6.9 ($\pm$ 1.27) ΔCa_s (○) $\Delta n = 2.6$ ($\pm$ 1.83) + 12.7 ($\pm$ 7.5) ΔCa_s where dose is units and the values in parentheses are S.D.

stimulated CaBP synthesis whereas the bone response was hardly noticeable for the first steroid and undetectable for the second.

Since the R epimer was more active in stimulating intestinal and bone responses, this compound was chosen for evaluation of its effectiveness in nephrectomized rats. The data from this experiment (Table 1) indicate that the loss of kidney prevents duodenal CaBP response. As it has been already reported [13] nephrectomy depressed serum calcium levels in control animals ($P < 0.001$).

DISCUSSION

From the experiments above described where a single dose of metabolites was administered to vitamin D-deficient rats on a low-calcium diet it is clear that 24R,25 and 24S,25-(OH)$_2$D$_3$ (1) stimulate CaBP synthesis and bone calcium mobilization (2) show a preferential action on the intestine. The R epimer appears more active in stimulating duodenal CaBP and bone calcium resportion than the S epimer. Furthermore, its biological activity requires renal metabolism.

In terms of CaBP the present data (Fig. 2) demonstrate that the intestinal response to the R epimer is parallel to that of 24S epimer for doses ranging from 130–2080 pmol. The coefficient of concentration ρ of the 24R epimer as compared to the 24S epimer is about 2. The CaBP response to increasing doses of 24R,25 and 24S,25-(OH)$_2$D$_3$ has not yet been reported in rats. However, since CaBP synthesis is highly correlated with the stimulation of calcium absorption by cholecalciferol [4] [14] it is interesting to compare our data to previous study [11] which reported that after a single injection of 650 pmol the S isomer was slightly less active than the R isomer on the intestinal calcium transport.

In terms of bone calcium mobilization, the bone response to the 24R epimer parallels that of the 24S epimer for doses ranging from 130 to 1040 pmol (Fig. 3). Again the coefficient of concentration ρ of 24R,25-(OH)$_2$D$_3$ as compared to 24S,25(OH)$_2$D$_3$ is about 2. This result is also supported by our time course study (Fig. 1). These data are not in complete agreement with the results of other authors [11]. Tanaka *et al.* reported that 650 pmol of R isomer sig-

Table 1. Response of duodenal calcium-binding protein (CaBP) and bone calcium mobilization (increment of serum calcium) to 24R,25-(OH)$_2$D$_3$ 24 h after intravenous steroid administration in intact and nephrectomized vitamin D-deficient rats

Rats	CaBP nmol Ca^{2+} bound/mg protein	Serum calcium (Ca_s) (mg/100 ml) hours after injection	
		0	24
Sham-operated:			
Vehicle (6)	4.6	6.3 $\pm$ 0.36	6.3 $\pm$ 0.43
Steroid 260 pmol (6)	13.3	6.2 $\pm$ 0.18	6.5 $\pm$ 0.18*
Nephrectomized:			
Vehicle (6)	2.0	6.1 $\pm$ 0.44	4.7 $\pm$ 0.61**
Steroid 1,040 pmol (7)	1.9	6.3 $\pm$ 0.17	4.4 $\pm$ 0.24**

Significance vs Ca_s at 0 h * $P < 0.02$, ** $P < 0.001$. The values in parentheses represent the number of rats.

nificantly stimulated the elevation of serum calcium concentration whereas at this dosage level the S isomer showed a barely significant activity.

Moreover, our results display a saturable bone response (Fig. 3) with the dose of 1040 pmol, whereas within the same dose range (1040–2080 pmol) the saturation of CaBP response was not apparent, especially with regard to the 24R epimer (Fig. 2).

It is of some interest to point out this discriminative response of intestine and bone to large doses of both epimers. We have previously reported [3] such a discrimination of the effectiveness on these target tissues with other vitamin D metabolites as 25-OH-D_3 and 1α,25-$(OH)_2D_3$. In these experiments the protocol was quite comparable to the one used in the present studies. A single injection of either 6.5 and 65 nmol of 25-OH-D_3 or 65 and 130 pmol of 1α,25-$(OH)_2D_3$ led to a maximal bone response and to an increased CaBP synthesis.

The present report demonstrates unequivocally that 24R,25 and 24S,25-$(OH)_2D_3$ are more effective in stimulating CaBP synthesis than in bone calcium mobilization (Fig. 4). Such an observation was reported in previous studies in which intestinal response was expressed by the measurement in vitro of the intestinal calcium transport [11].

From our results it is apparent that both stereoisomers exhibit no calcemic-response 12 h after their injection (Fig. 1). Moreover the delay after which 24R,25 and 24S,25-$(OH)_2D_3$ cause the maximal stimulation of bone calcium mobilization (24–48 h) is considerably more important than the one (12 h) found for 1α,25-$(OH)_2D_3$ (Fig. 1). These observations suggest that under our experimental conditions both epimers must be converted to 1α,24,25-$(OH_2D_3$ in the kidney before producing a calcemic response. Such a mechanism is also requisite to induce the intestinal response. Indeed a large dose (1040 pmol) of 24R epimer that is effective in stimulating CaBP production in intact animal is unable to initiate CaBP synthesis in nephrectomized rats (Table 1). These results are consistent with data previously reported [11] that demonstrated that nephrectomy prevents the intestinal calcium transport response to both isomers given at the high dose of 1.6 nmol.

Since the sole natural product was shown to be the R isomer [11] it is surprising to note that both stereoisomers are capable of stimulating CaBP synthesis and bone calcium mobilization. These results may suggest that in vitamin D and calcium-deficient rats the 25-OH-D_3-1-hydroxylase of the kidney is relatively unable to discriminate between the 24R and the 24S epimer. On the contrary a recent study [10] suggests the high selectivity of the 1-hydroxylase for the 24R epimer in the rat fed a low-phosphate, high-calcium and vitamin D-deficient diet.

Acknowledgements—This study was supported in part by INSERM Grant 73.5.063.5. We thank Dr A. W. Norman, Riverside, California, for the gift of 1,25-$(OH)_2D_3$ used in this investigation. We are grateful to A. Subrenat, N. Gouhier and A. Porteu de la Morandière for expert and devoted technical assistance.

REFERENCES

1. Bronner F. and Freund T.: *Am. J. Physiol.* **229** (1975) 689–694.
2. Boyle I. T., Omdahl J. L., Gray R. W. and DeLuca H. F.: *J. biol. Chem.* **248** (1973) 4174–4180.
3. Cuisinier-Gleizes P., Thomasset M. and Mathieu H.: In *Calcium et Maladies Endocrineinnes* (Edited by Laboratoire Sandoz) (1976) pp. 125–135.
4. Emtage J., Lawson E. and Kodicek E.: *Biochem. J.* **144** (1974) 339–346.
5. Freund T. and Bronner F.: *Am. J. Physiol.* **228** (1975) 861–869.
6. Henry H. L., Norman A. W., Taylor A. N., Hartenbower D. L. and Coburn J. W.: *J. Nutr.* **106** (1976) 724–734.
7. Holick M. F., Schnoes H. K., DeLuca H. F., Gray R. W., Boyle I. T. and Suda T.: *Biochemistry* **11** (1972) 4251–4255.
8. Redel J., Bazely N., Delbarre F. and Calando Y.: *C.r. hebd. Séanc. Acad. Sci., Paris* **283** (1976) 857–860.
9. Scatchard G.: *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
10. Tanaka Y., DeLuca H. F., Akaiwa A., Morisaki M. and Ikekawa N.: *Archs biochem. Biophys.* **177** (1976) 615–621.
11. Tanaka Y., DeLuca H. F., Ikekawa N., Morisanki M. and Koizumi M.: *Archs biochem. Biophys.* **170** (1975) 620–626.
12. Thomasset M., Redel J., Marche P. and Cuisinier-Gleizes P.: In *Proceedings of the Third Workshop on Vitamin D* (Edited by Walter de Gruyter, Berlin) (1977) pp. 619–621.
13. Walling M. W. and Kimberg D. V.: *Gastroenterology* **69** (1975) 200–205.
14. Wasserman R. H. and Taylor A. N.: *Science* **152** (1966) 791–793.