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CALCIUM-BINDING PROTEIN OF INTESTINE: INDUCTION BY BIOLOGICALLY SIGNIFICANT CHOLECALCIFEROL-LIKE STEROIDS *IN VITRO**

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SUMMARY

Vitamin D (cholecalciferol-like) steroids added to the culture medium induce a specific calcium-binding protein (CaBP)‡ in embryonic chick duodenum maintained in organ culture. Responses of the isolated duodenum faithfully mimic several other cholecalciferol-dependent responses observable *in vivo* attesting to their physiological significance. Therefore, this system provides a relevant bioassay, i.e. a physiological response (CaBP-induction) in a principal target organ, for the study of structure–activity relationships of biologically-significant cholecalciferol-like steroids.

The several cholecalciferol-like steroids normally circulating in the intact animal, including cholecalciferol itself, 25-hydroxycholecalciferol, 24R,25-dihydroxycholecalciferol and 1α,25-dihydroxycholecalciferol, induced CaBP biosynthesis *in vitro* at or below physiological concentrations. This clearly demonstrates the relative rather than absolute specificity of the intestine for cholecalciferol-like steroids in the induction of CaBP.

Maximal activity was observed with either 1α-hydroxycholecalciferol (not a native steroid), 1α,25-dihydroxycholecalciferol or 1α,24R,25-trihydroxycholecalciferol indicating a requirement for the 1α-hydroxyl function but suggesting the non-essentiality of the 25-hydroxyl function. The cis triene structure and an intact side-chain were also found to be essential to optimal activity. Preliminary studies with impure analogs suggest an absolute requirement for the 3β-hydroxyl group. The minimal structure inducing CaBP among naturally occurring steroids is cholecalciferol itself.

INTRODUCTION

Among several physiological actions of vitamin D (cholecalciferol), stimulation of the intestinal calcium absorptive mechanism has received considerable attention. At the molecular level, cholecalciferol acts as other hormonal steroids [1] and, in this case, there is *de novo* induction of a single new mRNA species coding for a calcium-binding protein (CaBP) in the intestine [2]. CaBP biosynthesis appears to be a concomittant of the action of cholecalciferol in increasing calcium absorption [3]. If not the sole agent of cholecalciferol-mediated calcium transport, CaBP is directly involved [4]. The exact mechanism of its action remains obscure.

An organ culture system using the embryonic chick duodenum has been developed which mimics the neonatal chick intestine *in vivo* in its responses to cholecalciferol [5]. It is particularly sensitive to cholecalciferol-like steroids added to the culture medium in terms of CaBP induction [5–7]. The present report examines quantitatively the structure-activity relationships of various biologically significant cholecalciferol-like steroids using this system.

METHODOLOGY

Organ Culture Technique. The culture technique has been described in detail elsewhere [5–8]. It consists basically of a system for maintenance of 20-day-old embryonic chick duodenum in organ culture in a defined medium. One of the distinct advantages of this system is the absence of CaBP from the embryonic intestine [9–11], providing a "zero CaBP" control. CaBP induction is undoubtedly *de novo* [12–14]. Measurement of CaBP is by means of a specific, sensitive radial immunodiffusion technique [5]. Other experimental details appear in the legends.

Steroids. Pure crystalline vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) were purchased from Sigma (St. Louis). Pure crystalline 25-hydroxycholecalciferol was the gift of Dr John Babcock, Upjohn Co., Kalamazoo, MI, U.S.A. DHT_3, 25-OH-DHT_3

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‡ Abbreviations and trivial names: vitamin D (cholecalciferol)-induced calcium-binding protein, CaBP; vitamin D_2 (ergocalciferol) 9,10-secoergosta-5,7,10(19), 22-tetraen-3β-ol; vitamin D_3 (cholecalciferol) 9,10-secocholesta-5,7,10(19)-trien-3β-ol; dihydrotachysterol$_3$ (DHT_3), 9,10-secocholesta-5,7-dien-3β-ol; 5,6-trans-cholecalciferol, 9,10-seco-(5E,7E)-5,7,10(19)-cholestatrien-3β-ol. All other abbreviations are self-explanatory on the basis of the position of hydroxyl functions or other modifications of the basic steroid nuclei.

and 5,6-trans-25-hydroxycholecalciferol were the gifts of Dr Philip Bell, Dunn Nutritional Labs, Cambridge, England. 5,6-trans-cholecalciferol was the gift of Dr H. F. DeLuca, University of Wisconsin, Madison, U.S.A. Side-chain analogs and the 3-deoxy derivatives were the gifts of Dr A. W. Norman, University of California, Riverside, U.S.A. Pure, crystalline synthetic 1α-hydroxycholecalciferol, 1α,25-dihydroxycholecalciferol, 1α,24R,25-trihydroxycholecalciferol and 24R,25-dihydroxycholecalciferol were the gifts of Dr M. Uskokovič of Hoffmann-LaRoche, Nutley, NJ, U.S.A.

The purity of the crystalline synthetic steroids was verified by U.V. absorption spectrometry and high pressure liquid chromatography (Waters Associates) using a 3.9 × 40 cm C^{18}-micro Bondapak column with an eluting solvent of MeOH:H_2O (85:15) at a flow rate of 1.5 ml/min (Maxwell, Villareale and Fullmer, unpublished). In this HPLC technique, baseline separation of all the steroids can be achieved in a single run with cholecalciferol itself being stripped off the column at the end with 100% MeOH. The purity of other steroids was assessed by t.l.c. [6] as well as U.V. spectrometry.

RESULTS

The results shown in Fig. 1 were obtained using pure, crystalline, synthetic steroids added to the culture medium at the concentrations indicated. The culture period was 48 h.

Several features of this data should be noted. First, the lowest effective levels inducing CaBP were equal to or considerably below circulating plasma levels reported by others. The exquisite sensitivity of the system should accordingly be emphasized. (Estimates of circulating cholecalciferol-like steroid concentrations have been reported as follows: cholecalciferol and 25-hydroxycholecalciferol, approximately 10^{-8} M [15, 16]; 1α,25-dihydroxycholecalciferol, approximately 10^{-10} M [16, 17]; 24R,25-dihydroxycholecalciferol, approximately 10^{-8} M [18]. The circulating level of 1α,24R,25-trihydroxycholecalciferol in normal animals is unknown. The 1α-hydroxycholecalciferol derivative has not been reported to occur naturally but it is of considerable biomedical interest.)

Second, the slopes of the response curves were statistically indistinguishable indicating that the same biochemical mechanism of induction was operative regardless of inducer. On the basis of these two features it is virtually certain that physiologically relevant responses were observed.

Third, the biological potencies of 1α-hydroxycholecalciferol, 1α,25-dihydroxycholecalciferol and 1α,24R,25-trihydroxycholecalciferol were statistically identical.

Relative potencies of all the cholecalciferol-like metabolites and analogs so far tested were calculated from the regression line for 1α,25-dihydroxycholecalciferol of Fig. 1 and the data presented in Table 1.

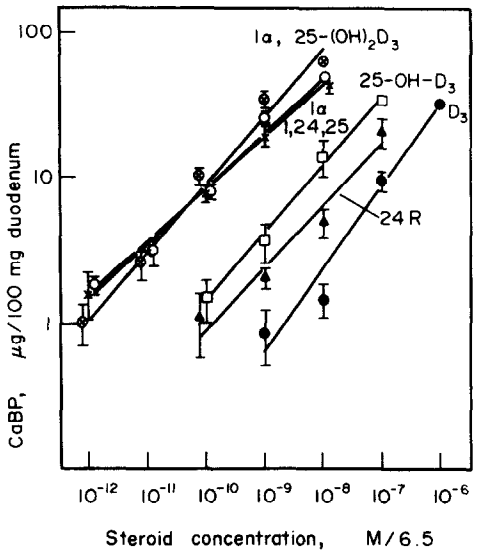

Fig. 1. CaBP induction by biologically significant cholecalciferol-like steroids in organ-cultured duodenum. Values are the mean ± S.E. of six duodena/point. The steroids used arrived as crystals in sealed ampules under argon from Dr Milan Uskokovič of Hoffmann-LaRoche. The lines plotted were obtained by regression analysis. All had correlation coefficients of ≥0.96 and slopes that did not differ significantly from each other. Symbols used: closed circles, cholecalciferol (D_3); closed triangles, 24R,25-dihydroxycholecalciferol(24R); closed stars, 1α,24R,25-trihydroxycholecalciferol (1,24,25); open squares, 25-hydroxycholecalciferol(25-OH-D_3); open circles, 1α-hydroxycholecalciferol (1α); open stars, 1α,25-dihydroxycholecalciferol (1α,25-$(OH)_2$-D_3).

For comparative purposes Table 1 shows the potency of the metabolite or analog relative to 1α,25-dihydroxycholecalciferol with a potency arbitrarily set at 10,000. It is obvious that cholecalciferol-like steroids varying in potency over a range of at least 10^4 can be tested in this system.

Because of the small amounts available, only one or two logarithmic concentrations of certain analogs could be tested and regression data could not always be obtained. However, the parallelism of the 'dose'–response curves shown in Fig. 1 indicates: first, a single biochemical mechanism of CaBP induction regardless of the chemical identity of the inducer; second, an enormous range over which the CaBP biosynthetic response to the inducer is log–log linear; and third, the only limiting factors for CaBP induction in this system appear to be steroid concentration and intrinsic potency. On these bases it was assumed that reasonably reliable estimates of relative potency could be obtained from this limited data. In every assay cholecalciferol was used as a reference control. Since the interexperimental response to this steroid is quite uniform it was unnecessary to normalize the CaBP concentrations induced by the test steroids. In cases where more than one concentration was tested, similar relative potencies were obtained when calculated as outlined in the legend of Table 1.

It is of some interest that the cholecalciferol-like

Table 1. Relative potencies of cholecalciferol metabolites and analogs in the induction of CaBP in the organ-cultured duodenum

Analog	Potency relative to 1α,25-dihydroxycholecalciferol[1]
1α,25-dihydroxycholecalciferol	10,000[2]
1α,24R,25-trihydroxycholecalciferol	10,000[2]
1α-hydroxycholecalciferol	10,000[2]
25-hydroxycholecalciferol	150[2]
25-hydroxydihydrotachysterol$_3$	75
24R,25-dihydroxycholecalciferol	30[2]
5,6-trans-25-hydroxycholecalciferol	30
Dihydrotachysterol$_3$	25
5,6-trans-cholecalciferol	15
Cholecalciferol	10[2]
24-homo-25-hydroxycholecalciferol	3 (tentative)[3]
Ergocalciferol	1
24-nor-25-hydroxycholecalciferol	1
3-deoxy-3α-methyl-1α-hydroxycholecalciferol	0.3 (tentative)[3]
3-deoxy-1α-hydroxycholecalciferol	0 (tentative)[3]
Side chain analogs with more than 1 carbon removed	0 (tentative)[3]

[1] Values were determined by use of the regression equation for 1α,25-dihydroxycholecalciferol activity in Fig. 1: $\log y = 0.47 \log x + 5.29$. That is, the value of y (CaBP concentration) induced by a given steroid was inserted into the equation and the value of x (1α,25-dihydroxycholecalciferol concentration) determined. The ratio of the actual concentration of the steroid used to the concentration of 1α,25-dihydroxycholecalciferol needed to achieve the same response was calculated. The potency of 1α,25-dihydroxycholecalciferol was arbitrarily set at 10,000 and the relative potencies of the other steroids were determined by dividing the calculated ratio into 10,000 (Table 1).

[2] Determined in one study using crystalline, synthetic steroids. Other values are reasonably accurate estimates from data obtained in previous studies (see Results).

[3] Values followed by the word 'tentative' were obtained using analogs which had degraded on shipping approximately 10–25%. These can be considered valid only to the extent that degradation products may not have been inhibitory.

steroids tested tend to cluster to some extent in terms of their potencies. For example, as already mentioned, the 1α-hydroxylated derivatives were equipotent regardless of other hydroxylations. The dihydroxylated derivatives were generally more potent than the monohydroxylated forms. Even minimal alteration of the side-chain resulted in considerably reduced or zero potency (ergocalciferol, 24-nor-25-hydroxycholecalciferol and 24-homo-25-hydroxycholecalciferol). Although the 3-deoxy derivatives degraded on shipping, it seems reasonable to suggest that the 3β-hydroxyl function is essential to biological activity. To a slight extent the 3α-methyl substitution appeared to provide some biological activity to the 3-deoxy derivative tested. Although pseudo 1-hydroxyl derivatives (the tachysterols and the 5,6-trans isomers) were more potent than cholecalciferol itself, it appears that optimal activity requires the cis triene structure as well as the 1α and 3β hydroxyl functions. This cannot be finally concluded, however, until the 5,6-trans-derivative of 1α-hydroxycholecalciferol has been tested. So far as is known this analog has not been synthesized.

DISCUSSION

Although induction of CaBP by cholecalciferol-like steroids represents an established, biologically-relevant response, the data obtained in this isolated intestinal system are not strictly comparable to data obtained in the intact animal for at least two reasons.

First, there is evidence that cholecalciferol-like steroids may undergo further metabolism *in vivo* prior to their action on the intestine, e.g. 1α-hydroxycholecalciferol is rapidly converted to 1α,25-dihydroxycholecalciferol [19], and this conversion may be responsible for the apparent equipotency of these two steroids *in vivo*. However, neither 25-hydroxylation of cholecalciferol nor 1α-hydroxylation of 25-hydroxycholecalciferol occurs to any detectable extent in the organ-cultured duodenum [6]. The possibility of conversion of 1α-hydroxycholecalciferol itself to 1α,25-dihydroxycholecalciferol in this system could not be directly tested. However, even if there were a 25-hydroxylase in the intact embryonic intestine which could effect this transformation, while unable to hydroxylate cholecalciferol itself, the data of Fig. 1 indicate that the conversion would have had to be quantitative. This seems unlikely since the 25-hydroxylase activity of intestinal homogenates is considerably less than that of liver, the major site of 25-hydroxylation *in vivo* [20]. Therefore, in terms of CaBP induction by the intact, functioning intestinal cell, the 1α-hydroxyl derivative appears to be the simplest structure possessing optimal biological potency.

Second, cholecalciferol-like steroids are probably

metabolized *in vivo* to inactive forms [21, 22]. Inactivation may depend on factors such as intestinal and other enzymes, which do not appear to function in the organ cultured duodenum. Further, storage and excretion occur *in vivo*. At least partly for these reasons, the relative potencies of 1α,25-dihydroxycholecalciferol and 25-hydroxycholecalciferol for intestinal calcium absorption *in vivo* are considerably lower [23, 24] than their relative potencies reported here. The absence of these factors in no way limits the utility of the *in vitro* system, but in fact greatly simplifies evaluation of new analogs. In addition the data obtained in this simple screening system can serve as a guide in the synthesis of cholecalciferol analogs for possible medical use. Of course, the final test of therapeutic value of new analogs requires experimental trials in intact animals and ultimately humans.

Another biomedical application is suggested by the data obtained with the synthetic analog, 24-nor-25-hydroxycholecalciferol. This steroid possesses very limited potency for CaBP induction, but has been reported to inhibit the liver 25-hydroxylase system [25] and, in that sense, has been proposed to be the first 'anti-cholecalciferol'. Whether this or other analogs, such as the recently reported 25-azacholecalciferol [26], interfere with CaBP induction by 1α-hydroxylated cholecalciferol derivatives at the intestinal level could be deduced using the organ-cultured duodenum. Local 'anti-cholecalciferols' could prove useful in counteracting cholecalciferol toxicity states. Attempts will be made to obtain potential anti-cholecalciferols' for testing.

Third, discrepancies exist between bioassay data, including the present data, and data on intestinal receptor specificity. For example, 1α,25-dihydroxycholecalciferol is 1000 times more potent than cholecalciferol in this system (Fig. 1). However, receptor binding studies show that cholecalciferol, if it competes at all with 1α,25-dihydroxycholecalciferol, does so only at concentrations many times greater than 1000-fold [27, 28]. Further, the 25-hydroxyl function appears to be unnecessary to optimal potency here, but appears to be essential to most efficient receptor binding [27, 28]. To some extent, at least, these observations may be explained on the basis of the relative solubility of the various steroids tested in the simple buffers used in the receptor studies. This particular problem may not be critical here because of the use of a neutral lipid solubilizer, crystalline albumin, in the culture medium and by the long (48 h) culture period.

Note should be taken of the lower activity of 24R,25-dihydroxycholecalciferol than 25-hydroxycholecalciferol (Fig. 1). It might be suggested that the 24R-hydroxyl function prevents the best fit with the *in situ* receptor but that this is overcome by the presence of the 1α-hydroxyl function.

There are some similarities between the relative potencies reported here and those reported for certain of the cholecalciferol-like steroids tested in fetal rat bone cultures [29]. CaBP has recently been detected in bone [30]. This observation may possibly explain the similar inactivity of 3-deoxy-1α-hydroxycholecalciferol in both systems [29], suggesting the need for the 3β-hydroxyl function for activity. The 3α-methyl function does seem to substitute to a slight extent for the missing hydroxyl group in the present studies (Table 1).

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REFERENCES

1. Haussler M. R. and McCain T. A.: Basic and clinical concepts related to vitamin D metabolism and action. *New Engl. J. Med.* **297** (1977) 974–983; 1041–1050.
2. Emtage J. S., Lawson D. E. M. and Kodicek R.: The response of the small intestine to vitamin D: Isolation and properties of chick intestinal polyribosomes. *Biochem. J.* **140** (1974) 239–247.
3. Wasserman R. H., Fullmer C. S. and Taylor A. N.: The vitamin D-dependent calcium-binding proteins. In *Vitamin D* (Edited by Lawson D. E. M.). Academic Press, New York (1978). In press.
4. Corradino R. A., Fullmer C. S. and Wasserman R. H.: Embryonic chick intestine in organ culture: stimulation of calcium transport by exogenous vitamin D-induced calcium-binding protein. *Archs Biochem. Biophys.* **174** (1976) 738–743.
5. Corradino R. A.: Embryonic chick intestine in organ culture: A unique system for the study of the intestinal calcium absorptive mechanism. *J. Cell Biol.* **58** (1973) 64–78.
6. Corradino R. A.: Embryonic chick intestine in organ culture: Response to vitamin D_3 and its metabolites. *Science* **179** (1973) 402–405.
7. Corradino R. A.: Embryonic chick intestine in organ culture: induction of CaBP as a simple, specific sensitive bioassay for 1α,25-$(OH)_2$-D_3. Fed. Proc. **36** (1977) 1047.
8. Corradino R. A.: Maintenance of embryonic chick duodenum in large scale organ culture. In *Tissue Culture Association Manual* (Edited by Evans V. J., Perry V. P. and Vincent, M. M.), Vol. 1, No. 2 pp. 71–74. Tissue Culture Association. Rockville, Maryland (1975).
9. Corradino R. A., Taylor A. N. and Wasserman R. H.: Appearance of vitamin D_3-induced calcium-binding protein (CaBP) in chick intestine during development. Fed. Proc. **28** (1969) 760.
10. Taylor A. N. and Wasserman R. H.: Vitamin D-induced calcium-binding protein: Comparative aspects in kidney and intestine. *Am. J. Physiol.* **223** (1972) 110–114.
11. Moriuchi S. and DeLuca H. F.: Metabolism of vitamin D_3 in the chick embryo. *Archs Biochem. Biophys.* **164** (1974) 165–171.
12. Corradino R. A.: 1,25-Dihydroxycholecalciferol: Inhibition of action in organ-cultured intestine by actinomycin D and α-amanitin. *Nature* **243** (1973) 41–43.
13. Emtage J. S., Lawson D. E. M. and Kodicek E.: Vitamin D-induced synthesis of mRNA for calcium-binding protein. *Nature* **246** (1973) 100–101.
14. Zerwekh J. E., Haussler M. R. and Lindell T. J.: Rapid enhancement of chick intestinal DNA-dependent RNA polymerase II activity by 1α,25-dihydroxy vitamin D_3, *in vivo*. *Proc. natn. Acad. Sci.* **71** (1974) 2337–2341.

15. Belsey R., DeLuca F. H. and Potts J. T.: Competitive binding assay for vitamin D and 25-OH vitamin D. *J. clin. Endocr. Metab.* **33** (1971) 554–557.
16. Hughes M. R., Baylink D. J., Gonnerman W. A., Toverud S. Y., Ramp W. K. and Haussler M. R.: Influence of dietary vitamin D_3 on the circulating concentration of its active metabolites in the chick and rat. *Endocrinology* **100** (1977) 799–806.
17. Eisman J. A., Hamstra A. J., Kream B. E. and DeLuca H. F.: A sensitive precise and convenient method for determination of 1,25-dihydroxy vitamin D in human plasma. *Archs Biochem. Biophys.* **176** (1976) 235–243.
18. Haddad J. G., Min C. and Walgate J.: Radioimmunoassay of human serum DBP, and competitive binding protein radioassay of 24,25-$(OH)_2$D. In *Vitamin D: Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism* (Edited by Norman A. W., Schaefer K., Coburn J. W., DeLuca H. F., Fraser D., Grigoleit H. G. and Herrath D. v.), pp. 463–471. Walter de Gruyter, New York (1977).
19. Zerwekh J. E., Brumbaugh P. F., Haussler D. H., Cork D. J. and Haussler M. R.: 1α-Hydroxy vitamin D_3: An analog of vitamin D which apparently acts by metabolism to 1α,25-dihydroxy vitamin D_3. *Biochemistry* **13** (1974) 4097–4102.
20. Holick S. A., Holick M. F., Tavella T. E., Schnoes H. K. and Deluca H. F.: Metabolism of 1α-hydroxyvitamin D_3 in the chick. *J. biol. Chem.* **251** (1976) 1025–1028.
21. Miravet L., Carre M., Ayigbede O. and Rasmussen H.: The effect of prednisolone upon intestinal metabolism and action of 25-hydroxy and 1,25-dihydroxyvitamin D_3. In *Vitamin D and Problems Related to Uremic Bone Disease* (Edited by Norman A. W., Schaefer K., Grigoleit H. G., Herrath D. v. and Ritz E.), pp. 59–65. Walter de Gruyter, New York (1975).
22. Kumar R., Harnden D. and DeLuca H. F.: Metabolism of 1,25-dihydroxy vitamin D_3: Evidence for side-chain oxidation. *Biochemistry* **15** (1976) 2420–2423.
23. Norman A. W., Procsal D. A., Okamura W. H. and Wing R. M.: Structure–function studies of the interaction of the hormonally active form of vitamin D_3, 1α,25-dihydroxy-vitamin D_3, with the intestine. *J. Steroid Biochem.* **6** (1975) 461–467.
24. DeLuca H. F. and Schnoes H. F.: Metabolism and mechanism of action of vitamin D. *A. Rev. Biochem.* **45** (1976) 631–666.
25. Johnson R. L., Okamura W. H. and Norman A. W.: Studies on the mode of action of calciferol. X. 24-nor-25-hydroxy vitamin D_3, an analog of 25-hydroxy D_3 having "anti-vitamin" activity. *Biochem. biophys. Res. Commun.* **67** (1975) 797–802.
26. Onisko B. L., Schnoes H. K. and DeLuca H. F.: 25-Azavitamin D_3: A vitamin D antagonist. *Fed. Proc.* **36** (1977) 913.
27. Brumbaugh P. F. and Haussler M. R.: 1α,25-dihydroxycholecalciferol receptors in intestine. I. Association of 1α,25-dihydroxy-cholecalciferol with intestinal mucosa chromatin. *J. biol. Chem.* **249** (1974) 1251–1257.
28. Procsal D. A., Okamura W. H. and Norman A. W.: Structural requirements for the interaction of 1α,25-$(OH)_2$-vitamin D_3 with its chick intestinal receptor system. *J. biol. Chem.* **250** (1975) 8382–8388.
29. Mahgaub A. and Sheppard H.: Effect of hydroxyvitamin D_3 derivatives on ^{45}Ca release from rat fetal bones *in vitro*. *Endocrinology* **100** (1977) 629–634.
30. Christakos S. and Norman A. W.: Vitamin D-dependent calcium binding protein in bone. *Fed. Proc.* **37** (1978) 408.