BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **225,** 203–208 (1996)
ARTICLE NO. 1154

Evidence for a 24R,25(OH)$_2$-Vitamin D$_3$ Receptor/ Binding Protein in a Membrane Fraction Isolated from a Chick Tibial Fracture-Healing Callus

Eun-Gyoung Seo,* Akira Kato,*[,1] and Anthony W. Norman*[,†,2]

Department of Biochemistry and †Division of Biomedical Sciences, University of California Riverside, California 92521

Received June 20, 1996

Earlier studies suggested a biological role for 24R,25(OH)$_2$-vitamin D$_3$ [24R,25(OH)$_2$D$_3$] in bone. This study was performed to investigate the possible existence of a binding protein/ receptor for 24R,25(OH)$_2$D$_3$ in tibial callus tissue. Previous reports indicated that imposition of a tibial fracture resulted in elevation of the chick renal 25(OH)D$_3$-24-hydroxylase during the healing process. The present study shows that a binding protein/receptor resides in a membrane fraction of the callus obtained 10 days after the fracture treatment; the receptor has a K_D value of 4.5 ± 0.8 nM (mean ± SEM). Using a steroid competition assay, where the *r*elative *c*ompetitive *i*ndex [RCI] of 24R,25(OH)$_2$D$_3$ was set to 100%, we found that the RCI of 24S,25(OH)$_2$D$_3$, 25(OH)D$_3$, and 1α,25(OH)$_2$D$_3$ were respectively 37.0 ± 8.2, 12.7 ± 3.9, and 2.0 ± 1.6 (mean ± SEM). These results indicate the existence of a saturable membrane receptor protein in the callus which has high ligand specificity for 24R,25(OH)$_2$D$_3$.

The second steroid vitamin D$_3$ is metabolized into two biologically active dihydroxylated metabolites, namely 1α,25(OH)$_2$-vitamin D$_3$ [1α,25(OH)$_2$D$_3$], which has been extensively studied (1), and 24R,25(OH)$_2$-vitamin D$_3$ [24R,25(OH)$_2$D$_3$], which has received less attention. Evidence has accumulated that 24R,25(OH)$_2$D$_3$ is an important vitamin D$_3$ metabolite which has functions distinct from those of 1α,25(OH)$_2$D$_3$ (2,3). Although 24R,25(OH)$_2$D$_3$ has a plasma level some 40-fold higher than that of 1α,25(OH)$_2$D$_3$ (4), its physiological role has not yet been clearly defined.

While several possible biological roles and sites of action have been suggested for 24R,25(OH)$_2$D$_3$, including regulation of parathyroid hormone release from the parathyroid gland (5,6), most studies concerning this vitamin D metabolite have focused on its possible actions on bone biology (7-11). The possible existence of a nuclear or cytosolic binding protein for 24R,25(OH)$_2$D$_3$ was reported in the chick parathyroid gland (12) and the long bone of rat epiphysis (13); however, there has been no general confirmation of these findings. Also several reports have described specific actions or accumulation of 24R,25(OH)$_2$D$_3$ in cartilage (11,14), or the bone fracture-healing callus tissue (15). With a rat costochondral chondrocyte culture model, Schwartz and Boyan reported that cells derived from resting cartilage respond primarily to 24R,25(OH)$_2$D$_3$ (8,16). In particular, these authors demonstrated actions of 24R,25(OH)$_2$D$_3$ on the matrix vesicles derived from the resting cartilage (17). Since matrix vesicles do not have a cell nucleus, this suggested that 24R,25(OH)$_2$D$_3$ was initiating biological responses via

[1] Dr. Kato is a Visiting Scientist from the Kureha Chemical Industry Co., Tokyo, Japan.
[2] Corresponding author. Fax: (909)787-4784. E-Mail: norman@ucrac1.ucr.edu.

0006-291X/96 $18.00

signal transduction pathways that did not involve a classical steroid hormone nuclear receptor. Nemere *et al.* (18) also suggested the possible existence of binding protein for 24R,25$(OH)_2D_3$ in the basal-lateral membrane of chick intestine.

The present study was carried out to investigate the possible existence of a 24R,25$(OH)_2D_3$ receptor/binding protein in the post nuclear pellet of the callus. Tissues were obtained 10 days after imposition of a chick tibial fracture and were based on the previous observation from this laboratory (submitted for publication) of a significantly elevated renal 25$(OH)D_3$-24-hydroxylase and serum 24,25$(OH)_2D_3$ level during this period of fracture healing. Our results indicate that there exists in chick tibial callus a highly specific membrane binding protein for 24R,25$(OH)_2D_3$ with characteristics typical of a receptor which distinguishes structurally related vitamin D_3 metabolites such as 25$(OH)D_3$, the unnatural isomer 24S,25$(OH)_2D_3$, and 1α,25$(OH)_2D_3$.

MATERIALS AND METHODS

Animals. Male White Leghorn chicks were obtained at hatching (Hyline International, Lakeview, CA) and were fed a vitamin D_3-replete diet (2000 IU vitamin D_3/kg diet) containing 0.6% calcium and 0.4% phosphate. When chicks were three weeks old, a tibial fracture surgery (approved by University of California Chancellor's Committee on Laboratory Animal Care: A-9412056-1) was performed on the right tibia: the chick was anesthetized with Metofane (methoxyflurane: Pitman-Moore, IL) inhalation and a skin incision ($\sim$1 cm) was made over the tibia. After the separation of overlying muscle tissues, a hole ($\sim$2 mm in diameter) was drilled with a dental burr in the diaphysis. The skin was then closed, sutured, and a complete fracture was made by applying light manual pressure. No form of fixation of the fracture was made. Ten days after fracture treatment, the birds were euthanized and the callus tissue was dissected and utilized for the various studies.

Tissue preparation. Immediately after the dissection of the callus, 10% homogenates in TED (10 mM Tris, 1.5 nM EDTA, 1 mM dithiothreitol at pH 7.4) were prepared using a Polytron (Brinkmann Instruments, Westburg, NY) briefly. The homogenate was centrifuged at 500 $\times$ g for 10 min (4°C) and the post nuclear supernatant was ultracentrifuged in a 60 Ti rotor (Beckmann Instruments) at 203,900 $\times$ g for 30 minutes. The resulting pellet was washed twice with TED by homogenizing (Potter-Elvehjem homogenizer) and centrifuging at 203,900 $\times$ g for 30 min. After the washes, the final post nuclear pellet was resuspended in TED and used for saturation analysis and steroid competition assay and described as follows.

Saturation analysis and Relative Competitive Index study of the membrane receptor. Saturation analysis was carried out over the range of 1-50 nM of [^{3}H]-24R,25$(OH)_2D_3$ (51.1 Ci/mmol: Kureha Chemical Co., Japan) in the presence or absence of 200-fold excess of nonradioactive 24R,25$(OH)_2D_3$, as described (19).

The relative ability of an analog to compete with [^{3}H]-24R,25$(OH)_2D_3$ for the binding to the putative receptor was measured by determination of the analog's *R*elative *C*ompetitive *I*ndex (RCI) value. RCI studies were performed as reported previously (20); in this assay, increasing amounts of nonradioactive 24R,25$(OH)_2D_3$, 24S,25$(OH)_2D_3$, 25$(OH)D_3$, or 1α,25$(OH)_2D_3$ were incubated with a saturating concentration of [^{3}H]-24R,25$(OH)_2D_3$ (16 nM). The reciprocal values of the percentage of maximally bound analogs were calculated and plotted as a function of the relative concentration of the analog and [^{3}H]-24R,25$(OH)_2D_3$. The slopes of the analog binding plots were then normalized to a standard curve obtained with nonradioactive 24R,25$(OH)_2D_3$ where the RCI value for 24R,25$(OH)_2D_3$ was set, by definition, to 100%.

RCI studies on various analogs for the binding to the serum vitamin D binding protein (DBP) (21) were also conducted at 15 nM [^{3}H]-24R,25$(OH)_2D_3$. A 1/1000 dilution of chick serum in TED buffer was used as a DBP source. In all experiments the bound and free [^{3}H]-24R,25$(OH)_2D_3$ were separated by using hydroxylapatite (22).

RESULTS AND DISCUSSION

Figure 1 presents a representative saturation analysis of the post nuclear membrane fraction obtained from callus tissue 10 days after imposition of a chick tibial fracture. The study utilized [^{3}H]-24R,25$(OH)_2D_3$ over the concentration range of 1 to 50 nM in the presence or absence of 200-fold excess nonradioactive ligand. Using a computer program (GraphPad Prism) for a one-site binding analysis, a K_D value of 3.98 nM and B_{max} of 75.8 fmol/mg protein were obtained for this particular experiment with a R^2 value greater than 98%. From 6 separate experiments, a K_D value of 4.49 $\pm$ 0.81 nM and B_{max} value of 50.0 $\pm$ 8.3 fmol/mg protein (mean $\pm$ SEM) were obtained.

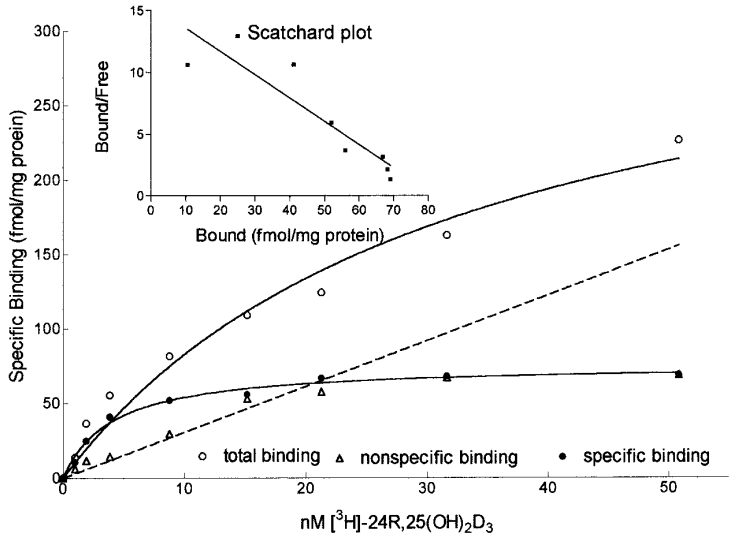

FIG. 1. Saturation analysis with [^{3}H]-24R,25(OH)$_2$D$_3$ of the post nuclear, membrane fraction obtained from chick tibial callus. The saturation analysis was performed on the post nuclear membrane fraction of the callus over the concentration range of 1-50 nM of [^{3}H]-24R,25(OH)$_2$D$_3$. The details are described in the Methods section. The data presented are representative results of 6 replicate experiments. A Scatchard transformation is presented in the inset. From six separate experiments, a K_D value of 4.49 ± 0.81 nM and Bmax of 50.0 ± 8.3 fmol/mg protein (mean ± SEM) was obtained.

The naturally occurring 24R,25(OH)$_2$D$_3$ has a hydroxyl located on carbon-24 in the R orientation. To further characterize the ligand specificity of this callus binding protein, the two related seco steroids, the unnatural 24S,25(OH)$_2$D$_3$ and the 24-deoxy 25(OH)D$_3$ were evaluated with respect to their ability to compete with the binding of [^{3}H]-24R,25(OH)$_2$D$_3$ by determination of their Relative Competitive Index (RCI). The results are summarized in Figure 2. When the RCI value for 24R,25(OH)$_2$D$_3$ was set to 100%, the RCI values (mean ± SEM) from 5 separate experiments for the other seco steroids were as follows: 37.0 ± 8.2 for 24S,25(OH)$_2$D$_3$, 12.7 ± 3.9 for 25(OH)D$_3$, and 2.0 ± 1.6 for 1α,25(OH)$_2$D$_3$. Clearly the callus membrane binding protein/receptor can distinguish between the natural 24R,25(OH)$_2$D$_3$ and the unnatural 24S,25(OH)$_2$D$_3$ and, as well, can detect the absence of the 24-hydroxyl [as in 25(OH)D$_3$].

One major concern relative to defining the ligand specificity of the putative membrane receptor for 24R,25(OH)$_2$D$_3$ is the possible contamination of the callus membrane fraction with the ubiquitous plasma vitamin D binding protein (DBP). Ten days after imposition of the tibial fracture, the callus is highly vascularized, and inevitably there are potentially significant amounts of DBP possibly present. As shown in Table 1, the RCI values of 24R,25(OH)$_2$D$_3$ and 24S,25(OH)$_2$D$_3$ for DBP are not significantly different from one another, while the RCI value of 25(OH)D$_3$ is much higher. We have previously reported an extensive study of the ligand specificities of DBP (23); of all the 37 naturally occurring metabolites of vitamin D$_3$, 25(OH)D$_3$ (24) has the highest Relative Competitive Index. Thus we conclude that the ligand specificity of the callus membrane binding protein (as reported in Fig. 2) is not attributable to the contaminating presence of DBP.

With this study, for the first time clear evidence for a specific membrane binding protein/receptor for 24R,25(OH)$_2$D$_3$ has been obtained in the chick tibial callus obtained 10 days after fracture treatment. The K_D value obtained is within the range of the physiological concentration of 24R,25(OH)$_2$D$_3$, which is 10-30 nM (25).

TABLE 1

Comparison of Relative Competitive Index Values for 24R,25$(OH)_2D_3$ and Related Compounds for the Vitamin D Binding Protein (DBP) in Serum with the Putative Membrane Binding Protein/Receptor Present in the Racture-Healing Chick Tibial Callus

Vitamin D metabolite or analog	RCI Serum DBP	RCI Callus membrane receptor
	(%)	(%)
24R,25$(OH)_2D_3$	100	100
24S,25$(OH)_2D_3$	100 ± 7	37.0 ± 8.2
25$(OH)D_3$	219 ± 23	12.7 ± 3.9
1α,25$(OH)_2D_3$	4.5 ± 1.8	2.0 ± 1.6

Steroid competition assays were performed as described in the Methods section to determine the RCI values. Values represent mean ± SEM from 3 or 5 separate experiments for DBP and callus membrane receptor, respectively.

The determination of the ligand specificity of the callus membrane receptor strongly suggests that this putative protein can distinguish small structural differences of vitamin D_3 metabolites or analogs. Furthermore, a different pattern of RCI values for the callus receptor from those for the D binding protein demonstrates that it is a separate binding protein from DBP, a ubiquitous protein (21) which frequently hampers study of the 24R,25$(OH)_2D_3$ receptor due to its high affinity for both 24R,25$(OH)_2D_3$ and 25$(OH)D_3$. Our results are consistent with the report (26) that only 24R,25$(OH)_2D_3$ is a biologically active metabolite, not its isomer 24S,25$(OH)_2D_3$, as shown by its low RCI value.

The results reported in this communication concerning the existence of a callus membrane binding protein/receptor which has high affinity for 24R,25$(OH)_2D_3$ but low affinity for 1α,25$(OH)_2D_3$ is similar to our previous report of the existence and biochemical purification of a membrane receptor for 1α,25$(OH)_2D_3$ which is present in the basal lateral membrane of the chick enterocyte (18). The 1α,25$(OH)_2D_3$ membrane receptor has a ligand specificity which is different from the nuclear receptor for 1α,25$(OH)_2D_3$ (27,28) and is known to be linked to the activation of rapidly appearing biological responses which are not dependent upon the involvement of the nuclear receptor for 1α,25$(OH)_2D_3$. It is possible that the two major circulating metabolites of vitamin D_3, which are both produced by the kidney, collectively mediate biological responses via interaction with separate receptors present in different target tissues which, in turn, activate different signal transduction pathways.

In conclusion, we report here, for the first time, strong evidence for the existence of a specific post nuclear, membrane binding protein/receptor for 24R,25$(OH)_2D_3$ in the healing callus tissue that results after imposition in the chick of a tibial fracture. The callus is a unique tissue that is generated as a consequence of a skeletal fracture; as such it represents a valuable bone biology model which recapitulates over a short time interval the ontogenic steps associated with maturation of bone. While the callus is heterogeneous and has a wide variety of cell types, based on the results obtained from prior studies of the biological effects of 24R,25$(OH)_2D_3$ on cartilage cells in tissue culture (8,16), it seems likely that the newly described membrane receptor for 24R,25$(OH)_2D_3$ is present in cartilage cells. Certainly further characterization and purification of this callus membrane protein will allow a more thorough understanding of the possible biological roles of 24R,25$(OH)_2D_3$ in bone.

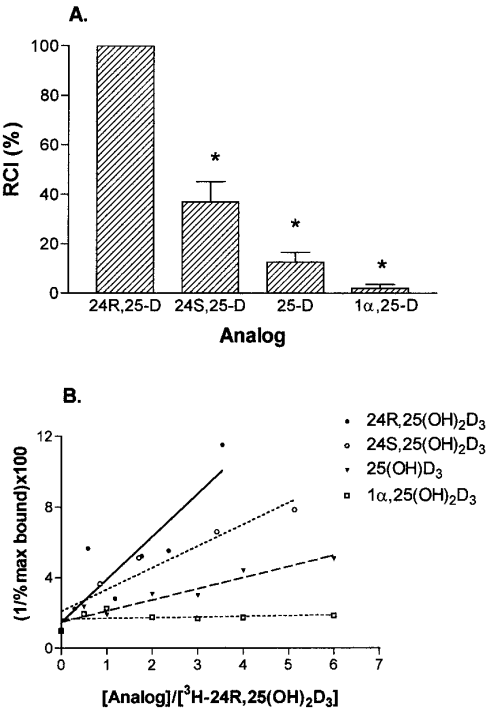

FIG. 2. *R*elative *C*ompetitive *I*ndex (RCI) values of vitamin D_3 analogs for competing with [^{3}H]-24R,25$(OH)_2D_3$ for binding to the callus post nuclear membrane binding protein. At 16 nM [^{3}H]-24R,25$(OH)_2D_3$, the RCI values for 24S,25$(OH)_2D_3$, 25$(OH)D_3$, and 1α,25$(OH)_2D_3$ were obtained when the value for 24R,25$(OH)_2D_3$ was set, by definition, to a value of 100%. Values represent mean ± SEM from 4-5 separate experiments (A): Asterisks (*) indicate significant difference from that of 24R,25$(OH)_2D_3$ at $p < 0.0001$. (B): The actual RCI plots from one typical experiment are also shown.

ACKNOWLEDGMENTS

The authors thank Ms. June Bishop for her advice and contributions to this project. Portions of this work were supported by grants from the USPHS Grant DK-09012-31(AWN) and the Biomedical Research Laboratories of the Kureha Chemical Industry Co., Tokyo, Japan.

REFERENCES

1. Bouillon, R., Okamura, W. H., and Norman, A. W. (1995) *Endocr. Rev.* **16,** 200–257.
2. Henry, H. L., and Norman, A. W. (1978) *Science* **201,** 835–837.
3. Matsumoto, T., Yamato, H., Okazaki, R., Kumegawa, M., and Ogata, E. (1992) *Proc. Soc. Exp. Biol. Med.* **200,** 161–164.
4. Norman, A. W., Roth, J., and Orci, L. (1982) *Endocr. Rev.* **3,** 331–366.
5. Canterbury, J. M., Lerman, S., Claflin, A. J., Henry, H. L., Norman, A. W., and Reiss, E. (1978) *J. Clin. Invest.* **61,** 1375–1383.
6. Chertow, B. S., Baker, G. R., Henry, H. L., and Norman, A. W. (1980) *Am. J. Physiol.* **238,** 384–388.
7. Henry, H. L., and Norman, A. W. (1991) *in* Disorders of Bone and Mineral Metabolism (Coe, F. L., and Favus, M. J., Eds.), pp. 149–162. Raven Press, New York, NY.
8. Schwartz, Z., Dean, D. D., Walton, J. K., Brooks, B. P., and Boyan, B. D. (1995) *Endocrinology* **136,** 402–411.
9. Norman, A. W., and Hurwitz, S. (1993) *J. Nutr.* **123** Suppl. 310–316.
10. Nakamura, T., Hirai, T., Suzuki, K., and Orimo, H. (1992) *Calcif. Tissue Int.* **50,** 74–79.
11. Seo, E.-G., Schwartz, Z., Dean, D. D., Norman, A. W., and Boyan, B. D. (1996) *Endocrine* (in press).
12. Merke, J., and Norman, A. W. (1981) *Biochem. Biophys. Res. Commun.* **100,** 551–558.
13. Somjen, D., Somjen, G. J., Weisman, Y., and Binderman, I. (1982) *Biochem. J.* **204,** 31–36.

14. Corvol, M., Ulmann, A., and Garabedian, M. (1980) *FEBS Letters* **116,** 273–276.
15. Lidor, C., Dekel, S., Hallel, T., and Edelstein, S. (1987) *J. Bone Joint Surgery* **69 no. 1,** 132–136.
16. Schwartz, Z., Brooks, B., Swain, L., Del Toro, F., Norman, A. W., and Boyan, B. D. (1992) *Endocrinology* **130,** 2495–2504.
17. Swain, L., Schwartz, Z., Caulfield, K., Brooks, B., and Boyan, B. D. (1993) *Bone* **14,** 609–617.
18. Nemere, I., Dormanen, M. C., Hammond, M. W., Okamura, W. H., and Norman, A. W. (1994) *J. Biol. Chem.* **269,** 23750–23756.
19. Wecksler, W. R., and Norman, A. W. (1980) *J. Biol. Chem.* **255,** 3571–3574.
20. Wecksler, W. R., and Norman, A. W. (1980) *in* Methods in Enzymology: Vitamins and Co-Enzymes, Vol. 67, pp. 494–500.
21. Cooke, N. E., and Haddad, J. G. (1989) *Endocr. Rev.* **10,** 294–307.
22. Bishop, J. E., Norman, A. W., Coburn, J. W., Roberts, P. A., and Henry, H. L. (1980) *J. Min. Electr. Metab.* **3,** 181–189.
23. Bishop, J. E., Collins, E. D., Okamura, W. H., and Norman, A. W. (1994) *J. Bone Miner. Res.* **9,** 1277–1288.
24. Henry, H. L., and Norman, A. W. (1984) *Ann. Rev. Nutr.* **4,** 493–520.
25. Horst, R. L., and Littledike, E. T. (1982) *Comp. Biochem. Physi.* **73B no. 3,** 485–489.
26. Somjen, D., Binderman, I., and Weisman, Y. (1983) *Biochem. J.* **214,** 293–298.
27. Dormanen, M. C., Bishop, J. E., Hammond, M. W., Okamura, W. H., Nemere, I., and Norman, A. W. (1994) *Biochem. Biophys. Res. Commun.* **201,** 394–401.
28. Norman, A. W., Okamura, W. H., Farach-Carson, M. C., Allewaert, K., Branisteanu, D., Nemere, I., Muralidharan, K. R., and Bouillon, R. (1993) *J. Biol. Chem.* **268,** 13811–13819.