Evidence for 24,25-dihydroxycholecalciferol receptors in long bones of newborn rats

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Several reports have appeared that suggest that 24,25-dihydroxycholecalciferol has a possible biological role in bone formation. We have utilized competition studies, saturation analysis, sucrose-density-gradient sedimentation and DEAE-cellulose chromatography to demonstrate that long bones of vitamin D-depleted newborn rats contain cytoplasmic and possibly nuclear receptors that bind 24,25-dihydroxycholecalciferol with specificity and high affinity ($K_d = 1.79$ nM). Sucrose-density-gradient analysis of the cytoplasmic 24,25-dihydroxycholecalciferol-binding component showed a single binding macromolecule for 24,25-dihydroxycholecalciferol with a sedimentation coefficient of 3.1 S. DEAE-cellulose chromatography showed a [3 H]24,25-dihydroxycholecalciferol-macromolecular complex that binds to DEAE-cellulose and elutes between 0.15 and 0.21 M-KCl. The finding of 24,25-dihydroxycholecalciferol receptors in long bones of newborn rats suggests a possible involvement of 24,25-dihydroxycholecalciferol in the metabolism of developing skeletal tissues.

1 α ,25-Dihydroxycholecalciferol, the active form of vitamin D (De Luca, 1976), like other steroid hormones, initiates its action by binding to cytoplasmic receptors in target cells. A cytoplasmic receptor for 1 α ,25-dihydroxycholecalciferol was first described in the intestinal mucosa (Tsai & Norman, 1973; Brumbaugh & Haussler, 1974). Similar receptor-like binding proteins have been identified in other tissues including bone (Kream *et al.*, 1977a,b; Manolagas *et al.*, 1979; Mellon & De Luca, 1980), kidney (Chandler *et al.*, 1979), parathyroid gland (Brumbaugh *et al.*, 1975), pituitary (Haussler *et al.*, 1980) and pancreas (Christakos & Norman, 1979). 24,25-Dihydroxycholecalciferol, another metabolite of vitamin D, has possible physiological functions in the formation of endochondral bone (Ornoy *et al.*, 1978; Endo *et al.*, 1980). Moreover, a specific nuclear binding of 24,25-dihydroxycholecalciferol has been demonstrated in cultured growth-plate chondrocytes (Corvol *et al.*, 1980). We now report the presence of specific cytoplasmic and nuclear receptors for 24,25-dihydroxycholecalciferol in developing endochondral bone of vitamin D-depleted newborn rats.

Experimental

Animals

Female Wistar rats (4 weeks old) were raised on a vitamin D-deficient diet containing 0.75% calcium

and 0.55% phosphorus and kept in darkness. At 130 days of age the females were mated with normal male rats and maintained during gestation and lactation periods on the same vitamin D-deficient diet. To ensure that the mother-rats were vitamin D-depleted, serum 25-hydroxycholecalciferol and 1 α ,25-dihydroxycholecalciferol concentrations were measured by competitive-binding radioassays (Eisman *et al.*, 1976; Weisman *et al.*, 1977). All animals had no detectable concentrations of 25-hydroxycholecalciferol and 1 α ,25-dihydroxycholecalciferol (<1.6 ng/ml and <5 pg/ml respectively). The females and their pups were killed at days 4 and 8 of lactation and at 50 days *post partum*.

Competitive-binding studies

Binding in vitro of radiolabelled vitamin D metabolites in slices of rat tissue. Slices of the epiphyseal region of newborn-rat tibia bones or the maternal duodenum were incubated in BGJ culture medium with 1.2 nM-[3 H]24,25-dihydroxycholecalciferol or 1.2 nM-[3 H]1 α ,25-dihydroxycholecalciferol in the presence or absence of a 100-fold excess of the appropriate non-radioactive vitamin D metabolite, i.e. 25-hydroxycholecalciferol, 24,25-dihydroxycholecalciferol or 1 α ,25-dihydroxycholecalciferol. The incubation was carried out at 37°C for 10 min. After incubation the slices were washed and homogenized (homogenizing buffer: 10 mM-

Tris/HCl buffer, pH 7.4, containing 250 mM-sucrose, 50 mM-dithiothreitol, 1.5 mM-EGTA and 3 mM-MgCl₂ in a motor-driven glass/Teflon homogenizer (for the duodenum) or a conical ground-glass homogenizer (for the tibiae). The homogenates were centrifuged at 800g for 10 min at 4°C to yield a nuclear pellet and a crude cytosol supernatant fraction. The pellets containing the nuclear proteins were washed twice, dissolved in 0.5 ml of 0.3 M-NaOH and both radioactivity and DNA content (Burton, 1956) were measured. The supernatant fraction was centrifuged at 39 000g for 10 min at 4°C and the resulting supernatant was used as the cytosol protein after measuring its protein content (Lowry *et al.*, 1951). Bound and free sterols were separated by a modification of the dextran-coated charcoal assay (Visser *et al.*, 1976). The specific binding of [³H]24,25-dihydroxycholecalciferol or [³H]1 α ,25-dihydroxycholecalciferol was calculated after subtraction of the non-specific binding in the presence of 100-fold excess of unlabelled metabolites.

Binding of [³H]24,25-dihydroxycholecalciferol and [³H]1 α ,25-dihydroxycholecalciferol in cytosol prepared from rat tissues. Cytosol was prepared from the epiphyses and diaphyses of newborn and of 50-day-old rat tibiae and from the maternal intestinal mucosa as described above. Portions of cytosol were incubated with 1.2 nM-[³H]24,25-dihydroxycholecalciferol or 1.2 nM-[³H]1 α ,25-dihydroxycholecalciferol in the presence or absence of 100-fold excess of the appropriate non-radioactive vitamin D metabolite, or progesterone or triamcinolone acetonide. Incubation was carried out for 90 min at 0°C. Bound and free sterols were separated with dextran-coated charcoal and the specific binding was calculated as described above.

Saturation plot and Scatchard analysis. Portions of cytosol prepared from epiphyses of newborn rat tibiae were incubated with increasing concentrations (0.6–24 nM) of [³H]24,25-dihydroxycholecalciferol with or without a 100-fold excess of the non-radioactive metabolite. Bound and free sterols were separated by dextran-coated charcoal. A saturation plot and Scatchard analysis were obtained from the total and specific binding data.

Sucrose-density-gradient analysis

Portions (0.5 ml) of cytosol prepared from the epiphyseal region of newborn rat tibiae were incubated with 1.2 nM-[³H]24,25-dihydroxycholecalciferol in the presence or absence of 100-fold excess of unlabelled 24R,25-dihydroxycholecalciferol for 90 min at 0°C. The bound and free sterols were separated by dextran-coated charcoal. Then the supernatant fractions were layered on 5–20% sucrose gradients prepared in 10 mM-Tris/HCl buf-

fer, pH 7.4, containing 500 mM-dithiothreitol, 1.5 mM-EGTA and 3 mM-MgCl₂. The gradients were centrifuged at 249 000g for 19 h at 0°C with the use of a Beckman L3-50 ultracentrifuge and an SW 50.1 rotor. Fractions (150 μ l) were collected from the bottom and counted for radioactivity. Sedimentation coefficients were estimated by comparison with ¹⁴C-labelled protein markers (bovine serum albumin, 4.0 S; aldolase, 7.8 S).

DEAE-cellulose chromatography

Portions (0.5 ml) of cytosol prepared from the epiphyseal region of newborn rat tibiae were incubated with 1.2 nM-[³H]24,25-dihydroxycholecalciferol for 90 min at 0°C and then applied to DEAE cellulose columns (0.5 g in 2 ml of buffer; Whatman DE-52), eluted with a linear salt gradient (0–0.6 M-KCl) in buffer (10 mM-Tris/HCl, pH 7.4, containing 50 mM-dithiothreitol, 1.5 mM-EGTA and 3 mM-MgCl₂) (Sherman *et al.*, 1978) and fractions (1.4 ml) were collected. Protein content (Bradford, 1976) and radioactivity were measured in each fraction. Salt concentrations were monitored by conductivity measurement.

Materials

Chemicals

25-Hydroxycholecalciferol, 24R,25-dihydroxycholecalciferol and 1 α ,25-dihydroxycholecalciferol were kindly provided by Dr. S. Edelstein, the Weizmann Institute of Science, Rehovot, Israel. Dithiothreitol, triamcinolone acetonide and progesterone were purchased from Sigma Chemical Co.

Radioactive materials and measurement of radioactivity

[23,24-³H]24,25-Dihydroxycholecalciferol (sp. radioactivity 77–82 Ci/mmol) and [23,24-³H]1 α ,25-dihydroxycholecalciferol (sp. radioactivity 90–110 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Radioactivity measurements were made in a Packard Tri-Carb automatic liquid-scintillation spectrometer no. 3380. Samples were counted for radioactivity in 3 ml of Hydro Luma scintillation solution. Counting efficiency varied from 47 to 49%.

Results

Competitive binding studies

The competition experiments (Tables 1 and 2) demonstrate that the epiphyseal and diaphyseal regions of newborn-rat bone possess specific receptors for 24,25-dihydroxycholecalciferol. 1 α ,25-Dihydroxycholecalciferol and 25-hydroxycholecalciferol were found to compete insignificantly for

Table 1. *Cytoplasmic and nuclear binding of [³H]24,25-dihydroxycholecalciferol and [³H]1α,25-dihydroxycholecalciferol in slices of epiphyses of newborn rat tibiae and the maternal intestinal mucosa*

The specific binding of [³H]24,25-dihydroxycholecalciferol or [³H]1α,25-dihydroxycholecalciferol was calculated after subtraction of the non-specific binding in the presence of 100-fold excess of non-radioactive metabolites. The control value of specific binding in the absence of competing metabolites was taken as 100%. The results given are means ± s.e.m. for three to eight determinations. For experimental details see the Experimental section.

Tissue	Competitor	Cytosol		Nuclei	
		[³ H]24,25-Dihydroxy-cholecalciferol binding (%) fmol/mg of protein)	[³ H]1α,25-Dihydroxy-cholecalciferol binding (%) fmol/mg of protein)	[³ H]24,25-Dihydroxy-cholecalciferol binding (%) fmol/mg of DNA)	[³ H]1α,25-Dihydroxy-cholecalciferol binding (%) fmol/mg of DNA)
Epiphyses of tibia bones (8-day-old rats)	—	100 (5.07 ± 1.50)	100 (0.07 ± 0.01)	100 (0.07 ± 0.01)	No binding
	25-Hydroxy-cholecalciferol	94.0 ± 14.7	100	100	—
	1α,25-Dihydroxy-cholecalciferol	83.0 ± 3.6	97.4 ± 6.5	98.0 ± 5.8	—
	24R,25-Dihydroxy-cholecalciferol	23.2 ± 3.1	97.6 ± 5.5	42.0 ± 0.6	—
Maternal intestine (duodenum)	—	100 (1.33 ± 0.26)	100 (5.02 ± 0.95)	100 (0.01 ± 0.02)	100 (0.14 ± 0.02)
	25-Hydroxy-cholecalciferol	84.2 ± 13.1	80.2 ± 12.4	61.4 ± 7.8	81.0 ± 4.6
	1α,25-Dihydroxy-cholecalciferol	95.5 ± 8.2	32.0 ± 4.2	86.0 ± 5.5	33.1 ± 7.4
	24R,25-Dihydroxy-cholecalciferol	88.3 ± 1.6	100	84.7 ± 15.8	67.6 ± 10.2

Table 2 *Binding of [³H]24,25-dihydroxycholecalciferol and [³H]1,25-dihydroxycholecalciferol in cytosol prepared from the epiphyses and diaphyses of newborn-rat tibiae and the maternal intestinal mucosa*

The specific binding of [³H]24,25-dihydroxycholecalciferol or [³H]1α,25-dihydroxycholecalciferol was calculated after subtraction of the non-specific binding in the presence of 100-fold excess of unlabelled metabolites. The control value of specific binding in the absence of competing sterols was taken as 100%. Values given are means ± s.e.m. For experimental details see the Experimental section.

Tissue	Competitor	[³ H]24,25-Dihydroxy-cholecalciferol binding (%) fmol/mg of protein)	[³ H]1α,25-Dihydroxy-cholecalciferol binding (%) fmol/mg of protein)
Epiphyses of tibia bones (4-day-old rats)	—	100 (462.0 ± 9.8)	No binding
	25-Hydroxycholecalciferol	96.2 ± 24.7	—
	1α,25-Dihydroxycholecalciferol	86.5 ± 7.9	—
	24R,25-Dihydroxycholecalciferol	7.6 ± 1.8	—
Diaphyses of tibia bones (4-day-old rats)	—	100 (205.6 ± 37.8)	100 (47.1 ± 9.8)
	25-Hydroxycholecalciferol	87.5 ± 5.2	74.0 ± 2.2
	1α,25-Dihydroxycholecalciferol	96.0 ± 2.3	45.7 ± 3.4
	24R,25-Dihydroxycholecalciferol	4.7 ± 0.6	73.5 ± 3.1
Diaphyses of tibia bones (50-day-old rats)	—	100 (276.0 ± 43.9)	100 (206.0 ± 21.5)
	25-Hydroxycholecalciferol	35.5 ± 5.2	97.2 ± 4.2
	1α,25-Dihydroxycholecalciferol	20.6 ± 5.6	29.7 ± 1.3
	24R,25-Dihydroxycholecalciferol	6.0 ± 1.1	86.0 ± 2.7
Maternal intestinal mucosa	—	No binding	100 (86.0 ± 14.2)
	25-Hydroxycholecalciferol	—	100
	1α,25-Dihydroxycholecalciferol	—	27.0 ± 3.6
	24R,25-Dihydroxycholecalciferol	—	95.5 ± 14.5

these binding sites. Similarly, a 100-fold excess of progesterone and triamcinolone acetonide failed to displace [^3H]24,25-dihydroxycholecalciferol from its cytoplasmic binding sites. In comparison, the binding of [^3H]24,25-dihydroxycholecalciferol to cytosol prepared from diaphyses of 50-day-old rat tibiae was non-specific.

No binding of [^3H]1,25-dihydroxycholecalciferol was found in the epiphyses of newborn rat tibiae and only a small amount of binding was detected in the diaphyses of these bones. However, a significant specific 1 α ,25-dihydroxycholecalciferol binding was found in cytosol prepared from diaphyses of 50-day-old rat tibia bones (Table 2). The maternal intestinal mucosa contains cytoplasmic and nuclear receptors for 1 α ,25-dihydroxycholecalciferol as previously described (Tsai & Norman, 1973; Brumbaugh & Haussler, 1974, 1975).

A saturation plot and Scatchard analysis of the specific binding of 24,25-dihydroxycholecalciferol to the cytoplasmic epiphyseal receptor revealed a plateau at 10 nM and indicated the presence of a

major class of binding sites with an apparent dissociation constant of 1.79 nM and an N_{max} value (maximal binding sites) of 2.79 pmol/mg of protein (Fig. 1).

Sucrose-density-gradient analysis

Sucrose-density-gradient analysis of the cytoplasmic [^3H]24,25-dihydroxycholecalciferol-binding component showed a single binding macromolecule for 24,25-dihydroxycholecalciferol with a sedimentation coefficient of approx. 3.1S (Fig. 2a). An excess of non-radioactive 24R,25-dihydroxycholecalciferol resulted in the disappearance of the 3.1S peak. No binding of [^3H]24,25-dihydroxycholecalciferol to the serum-generated approx. 6S-25-hydroxycholecalciferol-binding component (Kream *et al.*, 1977b) could be identified by sucrose-density-gradient analysis.

DEAE-cellulose column chromatography

The elution profiles of the DEAE-cellulose column chromatography (Fig. 2b) demonstrates a [^3H]24,25-dihydroxycholecalciferol-macromolecular complex that binds to DEAE-cellulose and elutes between 0.15 and 0.21 M-KCl. High-pressure liquid chromatography (Weisman *et al.*, 1979) of the chloroform/methanol extract of this complex yielded a single peak of radioactive material coinciding with standard 24R,25-dihydroxycholecalciferol.

Discussion

The data comprise three independent lines of evidence for the existence of a specific high-affinity approx. 3.1S cytoplasmic receptor for 24,25-dihydroxycholecalciferol in rat developing skeletal tissues. The competition studies also suggest the existence of specific nuclear binding of 24,25-dihydroxycholecalciferol in these tissues. The presence of specific cytoplasmic and nuclear receptors for 24,25-dihydroxycholecalciferol in the epiphyses and diaphyses of newborn rat bone suggests that the developing endochondral bone is a target organ for 24,25-dihydroxycholecalciferol and that this sterol acts as a steroid hormone (O'Malley & Schrader, 1976). Since the newborn epiphyseal and diaphyseal tissues contain substantial amounts of cartilage and relatively little bone, it is suggested that the chondrocyte is the cell type that contains the receptors for 24,25-dihydroxycholecalciferol. Recent studies that have shown a specific nuclear binding of 24,25-dihydroxycholecalciferol and its effects on DNA polymerase activity and proteoglycan synthesis in cultured growth-plate chondrocytes support this assumption (Corvol *et al.*, 1978, 1980). Furthermore, studies *in vivo* have shown the involvement of 24,25-dihydroxycholecalciferol in endochondral

Fig. 1. Saturation plot and Scatchard analysis of [^3H]24,25-dihydroxycholecalciferol binding in cytosol prepared from epiphyses of newborn rat tibia bones (a) Portions of cytosol were incubated with increasing concentrations of [^3H]24,25-dihydroxycholecalciferol in the presence (O) or absence (●) of a 100-fold excess of non-radioactive 24,25-dihydroxycholecalciferol. Bound and free sterols were separated by dextran-coated charcoal. (b) Scatchard analysis of specific binding.

Fig. 2. Sucrose density analysis (a) and DEAE-cellulose chromatography (b) of the cytoplasmic receptor for 24,25-dihydroxycholecalciferol in cytosol prepared from epiphyses of newborn rat tibia bones
(a) Portions (0.5 ml) of cytosol were incubated with 1.2 nM- $[^3\text{H}]24,25\text{-dihydroxycholecalciferol}$ in the presence (Δ) or absence ($\bullet$) of 100-fold excess of 24R,25-dihydroxycholecalciferol for 90 min at 0°C . Ultracentrifugation was carried out as described in the Experimental section. The arrows indicate the sedimentation of bovine serum albumin (4.0S) and aldolase (7.8S). (b) Portions (0.5 ml) of cytosol were incubated with 1.2 nM- $[^3\text{H}]24,25\text{-dihydroxycholecalciferol}$ for 90 min at 0°C and then applied to DEAE-cellulose columns (0.5 g; Whatman DE-52) and eluted with a linear salt gradient (0–0.6 M-KCl) in buffer. For experimental details see the Experimental section. ^3H ($\bullet$) was counted and protein ($\circ$) was determined in all fractions. Salt concentration (Δ) was monitored by conductivity measurements.

bone formation (Ornoy *et al.*, 1978; Endo *et al.*, 1980).

Previous studies (Kream *et al.*, 1977a; Manolagas *et al.*, 1979; Mellon & De Luca, 1980) have demonstrated the presence of $1\alpha,25\text{-dihydroxycholecalciferol}$ receptors in chick endochondral bone and in chick and rat calvaria bones. The present study has shown the existence of specific $1\alpha,25\text{-dihydroxycholecalciferol}$ binding in cytosol prepared from diaphyses of 50-day-old rat tibia, which are rich in bone cells, but not in cytosol prepared from tibiae of newborn rats. The presence of specific receptors for 24,25-dihydroxycholecalciferol is not exclusive to the rat or to developing skeletal tissues. We have identified a similar receptor (with a K_d of 0.21 nM and N_{max} of 2.35 pmol/mg of protein), in chondrocytes from cultures of chick-embryo limb-bud mesenchyme cells (D. Sömjen, G. J. Sömjen, A. Harell, G. L. Mechanic & I. Binderman, unpublished work). A recent study has also shown evidence for a 24,25-dihydroxycholecalciferol receptor (with a sucrose-gradient sedimentation coefficient of 3.2–3.4S) in the parathyroid gland of the rachitic chick (Merke & Norman, 1981).

References

- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Brumbaugh, P. F. & Haussler, M. R. (1974) *J. Biol. Chem.* **249**, 1258–1262
- Brumbaugh, P. F. & Haussler, M. R. (1975) *J. Biol. Chem.* **250**, 1588–1594
- Brumbaugh, P. F., Hughes, M. R. & Haussler, M. R. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4871–4875
- Burton, K. (1956) *Biochem. J.* **62**, 315–322
- Chandler, J. S., Pike, J. W. & Haussler, M. R. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1057–1063
- Christakos, S. & Norman, A. W. (1979) *Biochem. Biophys. Res. Commun.* **89**, 56–63
- Corvol, M. T., Dumontier, M. F., Garabedian, M. & Rappaport, R. (1978) *Endocrinology* **102**, 1269–1274
- Corvol, M., Ulmann, A. & Garabedian, M. (1980) *FEBS Lett.* **116**, 273–276
- De Luca, H. F. (1976) *J. Lab. Clin. Med.* **87**, 7–26
- Eisman, J. A., Hamstra, A. J. & De Luca, H. F. (1976) *Science* **193**, 1021–1023
- Endo, H., Kiyoki, M., Kawashima, K., Naruchi, T. & Hashimoto, Y. (1980) *Nature (London)* **286**, 262–265
- Haussler, M. R., Manolagas, S. C. & Deftos, L. J. (1980) *J. Biol. Chem.* **255**, 5007–5010
- Kream, B. E., Yamada, S., Schnoes, H. K. & De Luca, H. F. (1977a) *J. Biol. Chem.* **252**, 4501–4505

- Kream, B. E., Jose, M., Yamada, S. & De Luca, H. F. (1977b) *Science* **197**, 1086–1088
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Manolagas, S. C., Taylor, C. M. & Anderson, D. C. (1979) *J. Endocrinol.* **80**, 35–39
- Mellon, W. S. & De Luca, H. F. (1980) *J. Biol. Chem.* **255**, 4081–4086
- Merke, J., & Norman, A. W. (1981) *Biochem. Biophys. Res. Commun.* **100**, 551–558
- O'Malley, B. W. & Schrader, W. T. (1976) *Sci. Am.* **234**, 32–44
- Ornoy, A., Goodwin, D., Noff, D. & Edelstein, S. (1978) *Nature (London)* **276**, 517–519.
- Sherman, M. R., Somjen, G. J. & Pickering, L. A. (1978) *Program of the 60th Annual Meeting of the Endocrine Society, Miami*, p. 132 (abstr. 115)
- Tsai, H. C. & Norman, A. W. (1973) *J. Biol. Chem.* **248**, 5967–5975
- Visser, T. J., Bernard, H. F., Doctor, R. & Ittenman, G. (1976) *Acta Endocrinol. (Copenhagen)* **82**, 98–104
- Weisman, Y., Reiter, E. & Root, A. (1977) *J. Pediatr.* **91**, 904–907
- Weisman, Y., Harell, A., Edelstein, S., David, M., Spirer, Z. & Golander, A. (1979) *Nature (London)* **281**, 317–319