Purification, immunological and biochemical characterization of rat 28 kDa cholecalciferol-induced calcium-binding proteins)

Identity between renal and cerebellar cholecalciferols

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The cholecalciferols are intracellular vitamin D-dependent calcium-binding proteins. High concentrations of 28 kDa cholecalciferols have been found in the kidneys and cerebella of birds and mammals. However, whereas the synthesis of the renal protein is vitamin D-dependent, that of the cholecalciferol in the cerebella of young growing chicks and rats is apparently not. In the present study a range of immunological, physicochemical and structural characteristics of renal and cerebellar cholecalciferols isolated from a single species, the rat, has been examined to ascertain what, if any, differences there are between them, other than the vitamin D-dependence. Both proteins behaved in exactly the same way during purification and showed complete immunocross-reactivity in Ouchterlony double immunodiffusion and radioimmunoassay. No difference could be detected between them on electrophoresis under denaturing conditions or on two-dimensional isoelectric focusing/electrophoresis. Their amino acid compositions were very similar, as were their u.v.-absorption spectra and their chymotryptic and tryptic peptide maps. The *N*-terminal sequence was found to be Gly-Gly-Val-Ser... for both cholecalciferols. We have thus been unable to show any significant difference between the two proteins and suggest that the 28 kDa cholecalciferols of the rat cerebellum and kidney are extremely similar, if not identical.

INTRODUCTION

Calcium-binding proteins (CaBPs) are present in the cells of many plants and animals and share a number of common structural features (Goodman, 1980). Some of these proteins, such as calmodulin, are almost ubiquitous, whereas others have a much more restricted distribution. Among the latter are the cholecalciferols (cholecalciferol-induced CaBPs). The first cholecalciferol was detected in, and subsequently isolated from, the chick intestine (Wasserman & Taylor, 1966). It has since been found in a variety of other chick tissues and in a number of other species (Wasserman & Feher, 1977).

Although all the tissues of the chick seem to contain the same cholecalciferol (M_r 28000), mammals possess two biochemically and immunologically distinct types of protein (Thomasset *et al.*, 1982): a small 9 kDa cholecalciferol, which is most abundant in the duodenum, and larger 28 kDa cholecalciferols, found mainly in the kidney and cerebellum. The 28 kDa cholecalciferols have some limited immunological similarities to the chick cholecalciferol (Thomasset *et al.*, 1982).

One of the basic characteristics of the cholecalciferol as a whole is the dependence of their synthesis on vitamin D. Although this has been well documented in the growing rat for the renal 28 kDa cholecalciferol, neither we (Thomasset *et al.*, 1982), nor Taylor (1974) in his study of the chick cerebellum, were able to demonstrate such

a dependence for the cerebellar protein. As a result, the relationship between the two proteins remains unclear. Cholecalciferols of M_r 28000 have been isolated from mammalian kidney and cerebellum by several workers (Staun *et al.*, 1984; Sonnenberg *et al.*, 1984; Baimbridge *et al.*, 1982), but no systematic comparison of the two proteins has been undertaken. The present study compares renal and cerebellar 28 kDa cholecalciferols from the same species, the rat. On the basis of a number of common characteristics, their identical behaviour during purification and in three electrophoretic systems, immunological reactivity, amino acid compositions, peptide maps and *N*-terminal sequences, the results obtained support the concept that the rat cerebellar and renal 28 kDa cholecalciferols are very similar, if not identical.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (4 weeks old; Charles River) were killed and the kidneys and cerebella immediately removed. Decapsulated renal cortex and cerebella were quick-frozen in liquid N_2 and stored at $-20^\circ C$ until used.

Sephadex G-25, G-100, Polybuffer 74 and Polybuffer Exchanger 94 were purchased from Pharmacia. Aprotinin, trypsin and chymotrypsin were from Sigma; $Na^{125}I$ was

Abbreviations used: CaBP, calcium-binding protein; SDS, sodium dodecyl sulphate; PEG, poly(ethylene glycol); DABITC, 4-*NN'*-dimethylaminoazobenzene-4'-isothiocyanate; DABTH, 4-*NN'*-dimethylaminoazobenzene-4'-isothiazolinone; PITC, phenyl isothiocyanate.

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from Amersham International; 4-*NN'*-dimethylaminoazobenzene 4' isothiocyanate (DABITC), phenylisothiocyanate (PITC), pyridine, dimethylallylamine and *n*-heptane were from Fluka A.G., Chelex-100 was from Bio-Rad, and polyamide sheets (2.5 cm × 2.5 cm, F1700) were from Schleicher and Schull. All other chemicals were reagent-grade. Calmodulin was a gift from Professor J. Demaille (CNRS, Montpellier, France).

Methods

Purification. Essentially the same procedure was used for kidney and cerebella. All steps were carried out at 4 °C unless otherwise noted.

Preparation of cytosol fraction. A 30 g portion of tissue was homogenized in 4 vol. of Tris/HCl buffer (14 mM-Tris/HCl/0.12 M-NaCl/3 mM-KCl, pH 7.4) containing aprotinin (0.01–0.02 units/ml) with a motor-driven Potter–Elvehjem homogenizer. The homogenates were centrifuged at 100 000 *g* for 1 h. The resulting clear supernatant solution (cytosol) was heated to 65 °C for 10 min, cooled on ice and the heat-precipitated proteins removed by centrifugation at 10 000 *g* for 15 min.

Gel-filtration chromatography. Heat-treated cytosols were chromatographed on a column (2.6 cm × 100 cm) of Sephadex G-100 equilibrated with 0.02 M-ammonium acetate, pH 7.2. Proteins were eluted with the same buffer at a flow rate of 5 ml/h per cm². Calcium-binding activity was determined by using the Chelex assay previously described (Freund & Bronner, 1975). Fractions were pooled and freeze-dried.

Chromatofocusing. Further purification was achieved by chromatofocusing. Freeze-dried protein was dissolved in 0.025 M-imidazole/HCl, pH 7.0, containing 1 mM-CaCl₂, and applied to a column (1 cm × 30 cm) of Polybuffer Exchanger 94 equilibrated in the same buffer. Elution was carried out with Polybuffer 74, at a flow rate of 15 ml/h per cm², producing a pH gradient from 7 to 4. Cholecalciferol was detected immunologically with an antibody to human cerebellar 28 kDa cholecalciferol (Thomasset *et al.*, 1982), and positive fractions were pooled.

Gel-permeation h.p.l.c. The final purification step was h.p.l.c. Protein was dissolved in 0.01 M-Tris/HCl/0.15 M-NaCl, pH 7.2, and aliquots (500 µg in 100 µl) were injected on to a Waters I125 column fitted to a Waters chromatograph and eluted with the same buffer, at room temperature, at a flow rate of 0.4 ml/min. Fractions of volume 0.2 ml were collected, monitored at 280 nm and the 28 kDa cholecalciferol detected immunologically.

Immunological methods. Antisera. New Zealand white rabbits were injected intradermally with 200 µg of pure renal 28 kDa cholecalciferol in complete Freund's adjuvant. The initial injection was followed by six monthly injections, each of 100 µg of cholecalciferol. Rabbits were bled by cardiac puncture 7 days after the 6th and 7th booster injections.

Ouchterlony (1967) double diffusion. Agarose gels (1.5% in 0.02 M-phosphate buffer, pH 7.4, containing 0.1 M-NaCl) were prepared and samples of antiserum and test solutions placed in appropriate wells. The gels were incubated for 16 h at 4 °C and stained with Amido Black [0.6% in 2% (v/v) acetic acid].

Radioimmunoassay. Renal 28 kDa cholecalciferol (4 µg) was iodinated by the lactoperoxidase method (Marche *et al.*, 1977) and the labelled cholecalciferol purified by

successive chromatography on Sephadex G-25 and G-100 in 0.02 M-Tris/HCl, pH 7.2, containing 0.5% bovine serum albumin. The radioimmunoassay was carried out as described previously (Thomasset *et al.*, 1982). The standard was pure renal 28 kDa cholecalciferol (1–40 ng/tube). All standards, unknown samples, ¹²⁵I-cholecalciferol and antisera (final dilution 1:15 000) were diluted in 0.02 M-phosphate buffer, pH 7.4, containing 0.5% bovine serum albumin.

Physicochemical characteristics. Electrophoresis. SDS/polyacrylamide-gel electrophoresis was carried out under denaturing conditions as described by Laemmli (1970), with 15% (w/v) polyacrylamide-gel slabs. Two-dimensional isoelectric focusing–electrophoresis was performed by the method of Righetti *et al.* (1978). Each gel slot was loaded with ¹²⁵I-cholecalciferol and 100 µg of unlabelled cholecalciferol. Proteins were stained with Coomassie Brilliant Blue R-250. Gels were vacuum-dried and autoradiographed by using Kodak X-Omat AR film.

Amino acid analysis. Samples of 28 kDa cholecalciferols (5 nmol) were hydrolysed *in vacuo* in 6 M-HCl containing 0.05% 2-mercaptoethanol for 24, 48 and 72 h at 120 °C. Cysteine was determined after aminoethylation (Raftery & Cole, 1966) and hydrolysis for 24 h in 6 M-HCl. All analyses were performed on a Chromatospek J 180 (Rank–Hilger) analyser, a single-column procedure and 1 h program being used.

Peptide mapping. Tryptic and chymotryptic digestions of 3 nmol of cholecalciferol were done in 0.02 M-NH₄HCO₃, pH 8.2, in the presence and absence of 0.1 mM-Ca²⁺ for 16 h at 37 °C, an enzyme/protein ratio of 1/100 (w/w) being used. Digestion was stopped by freeze-drying. The resulting peptides were chromatographed on a RP-300 column (Brownlee Laboratories) fitted to a Gilson chromatograph. Elution was performed with a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid/water at a flow rate of 2 ml/min. The effluent was monitored at 220 nm.

N-Terminal sequencing. Manual Edman degradation with DABITC and PITC as described by Chang *et al.* (1978) was used. Cholecalciferol (5 nmol) was treated with DABITC and PITC and the amino acid DABTH derivatives were separated and identified by t.l.c. on polyamide sheets.

RESULTS AND DISCUSSION

Purification

Heat precipitation was included in the purification, since the vitamin D-dependent CaBPs are heat-stable (Bredderman & Wasserman, 1974). Its use resulted in the removal of approx. 70% of the total protein and a 30% loss of cholecalciferol as detected by radioimmunoassay; a similar result was recently reported by Staun *et al.* (1984) for human renal cholecalciferol.

28 kDa cholecalciferol from kidney and cerebellum were eluted in identical positions during each of the chromatographic stages of purification. Both were eluted with an apparent *M_r* of 28 000 on Sephadex G-100 gel-filtration chromatography and at pH 4.5 during chromatofocusing in the presence of 1 mM-Ca²⁺ (Fig. 1). They also had the same retention times on gel-permeation h.p.l.c. (Fig. 2). The yields, expressed as mg of cholecalciferol/100 g of fresh tissue, were 2 and 6.5 for

Fig. 1. Elution profile of CaBPs from the Polybuffer Exchanger 94 chromatofocusing column

Partially purified rat cholecalciferols were applied on a Polybuffer Exchanger 94 column. The start buffer was 25 mM-imidazole/HCl (pH 7)/1 mM- CaCl_2 . Elution was performed with Polybuffer 74, pH 4, with 1 mM- CaCl_2 at a flow rate of 12 ml/h producing a pH gradient from 7 to 4. Fractions (1 ml) were tested for immunoreactive CaBP. —, Renal cholecalciferol (A_{280}); ---, cerebellar cholecalciferol (A_{280}); ·····, CaBP immunoreactivity; ·····, pH gradient.

Fig. 2. Gel-permeation h.p.l.c.

Proteins (500 μg) were injected into the h.p.l.c. column and elution was at a flow rate of 0.4 ml/min. —, Renal cholecalciferol; ---, cerebellar cholecalciferol. CaBP was detected by immunoreactivity and corresponds to the main peak eluted at 20 min.

kidney and cerebellum respectively. The behaviour of these two rat cholecalciferols compares well with that of the large CaBP recently isolated from human kidney (Staun *et al.*, 1984). They have similar apparent M_r values, and the elution of all three at pH 4.5 during chromatofocusing indicates that they are acidic proteins.

Although several authors (Staun *et al.*, 1984; Pasini &

Fig. 3. SDS/polyacrylamide-gel electrophoretic analysis of renal and cerebellar cholecalciferols after the final step of purification

Lane 1, cerebellar CaBP (20 μg); lane 2, renal CaBP (20 μg).

Christakos, 1984) have reported satisfactory purification of cholecalciferols after gel filtration and ion-exchange or isoelectric focusing, we found that the preparation of pure cholecalciferol from rat cerebellum and kidney required an additional step: gel-exclusion h.p.l.c. (Fig. 2). Although the yields from this final step were low, the chromatography was extremely rapid and reproducible and could be used in a preparative procedure. The final products obtained from both kidney and cerebellum appeared as single band on SDS/polyacrylamide-gel electrophoresis (Fig. 3), migrating with apparent M_r values of 26000.

Immunology

When homogenates of kidney and cerebellum were tested against the antiserum raised to purified renal cholecalciferol by Ouchterlony immunodouble diffusion (Fig. 4), a single precipitin band was seen for each cholecalciferol. These bands were perfectly fused, with no trace of spurs, suggesting that a single immunological species reacting to the cholecalciferol antiserum is present in both tissues.

Such immunocross-reactivity between the rat 28 kDa cholecalciferols was also supported by the radioimmunoassay data (Fig. 5). The curves for cerebellar and renal extracts are parallel with the standard curve constructed by using renal cholecalciferol. The present data also provide some evidence for the immunological identity of 28 kDa cholecalciferols from various mammals (pig, mouse and rat) (Fig. 5). In contrast, other intracellular CaBPs, such as rat 9 kDa cholecalciferol (Figs. 4 and 5), rat skin CaBP,

Fig. 4. Ouchterlony double immunodiffusion

The centre well contained undiluted anti-(rat kidney CaBP) immunoserum. Wells A and B contained respectively 0.3 and 0.6 μg of partially purified renal CaBP. Wells C, D and E contained 0.5, 1 and 2 μg of partially purified rat cerebellar CaBP. Wells F, G and H contained 0.5, 1 and 2 μg of pure rat duodenal CaBP.

Fig. 5. Radioimmunoassay standard curve for 28 kDa CaBP

●, Rat renal CaBP; ▲, dilutions of human, rat and pig renal homogenates; ■, dilutions of human, rat and pig cerebellum homogenates.

bovine brain S100 protein and calmodulin, showed no cross-reactivity.

The sensitivity of the assay, with a minimum measurable level of 1.0 ng/tube, is similar to other published assays for the 9 kDa and 28 kDa cholecalcins (Marche *et al.*, 1977; Thomasset *et al.*, 1982).

Physicochemical characteristics

Electrophoresis. A detailed analysis of the electrophoretic properties in the presence and absence of 1 mM- Ca^{2+} was done by using a two-dimensional system of electrophoresis and electrofocusing with a pH gradient from 3.5 to 10 (Fig. 6). The experiment was carried out by running, in a single apparatus, tandem gels containing ^{125}I -labelled renal cholecalcins and unlabelled cerebellar protein, one in the presence of calcium (Fig. 6c) and the other in its absence (Figs. 6a–6b). Titration curves obtained after protein staining (cerebellar cholecalcins)

and autoradiography (renal cholecalcins) were completely superimposable. Control experiments in which labelled and unlabelled kidney cholecalcins samples were run together indicated no influence of the labelling on the electrophoretic behaviour (results not shown). Fig. 6d is a composite tracing showing the migration of renal cholecalcins in the presence and absence of Ca^{2+} . Ca^{2+} simultaneously shifts the zero-mobility point from 4.3 to 4.5 and diminishes the electrophoretic mobility in the alkaline part of the gel. This was also confirmed by chromatofocusing where the peak was eluted at a pH of 4.3 when we omit Ca^{2+} (results not shown). A pI of 4.5 in the presence of Ca^{2+} was thus confirmed for both renal and cerebellar cholecalcins.

Staun *et al.* (1984) observed no such Ca^{2+} -dependent shift in the pI for human renal cholecalcins, but there have been several reports of such a shift for the smaller 9 kDa cholecalcins from mammalian intestine (Hitchman *et al.*, 1973; Delorme *et al.*, 1982). The former group used this property in the purification of hog intestinal cholecalcins.

The decreased electrophoretic mobility is consistent with the masking of acidic residues which occurs on Ca^{2+} binding, as has previously been described for other calcium-binding proteins (Klee *et al.*, 1979) and this, in turn, has been related to the interaction of Ca^{2+} with carboxy groups (Williams, 1980).

Structural characteristics/chemical properties. Table 1 shows the amino acid compositions of rat renal and cerebellar cholecalcins and, for comparison, chick intestinal CaBP (Bredderman & Wasserman, 1974). All three proteins are characterized by high aspartic acid and glutamic acid contents. The overall amino acid contents are remarkably similar, with the possible exceptions of glycine and cysteine, the contents of which are lower in the chick CaBP, as might be expected, since the rat and chick cholecalcins show partial immunocross-reactivity (Thomasset *et al.*, 1982). A comparison of the values obtained in the present study with the compositions recently published for two newly described bovine cerebral calcium-binding proteins, caligulin (Waisman *et al.*, 1983; Baudier *et al.*, 1985), and CBP-18 (Manolan *et al.*, 1984), indicates that there may be a considerable number of common structural features within this group of proteins.

The u.v.-absorption spectra (not shown) for renal and

Fig. 6. Two-dimensional isoelectric focusing (I.e.f.)/electrophoresis (E.i.)

These were performed on polyacrylamide-gel slabs, with 7% (w/v) acrylamide with 2% (w/v) Ampholines of pH range 3.5–10. (a) and (b) correspond to a single gel run in the absence of Ca^{2+} ; (a) protein staining of cerebellar cholecalciferol; (b) autoradiograph of ^{125}I -labelled renal cholecalciferol; (c) corresponds to the autoradiograph of ^{125}I -labelled renal cholecalciferol in the presence of 1 mM- Ca^{2+} ; (d) is a schematic comparison of the two autoradiographs —, no Ca^{2+} ; ----, 1 mM- Ca^{2+} .

cerebellar cholecalciferols appear identical, both having a maximum at 280 nm, together with a characteristic tryptophan shoulder at 290 nm. The identity of the two spectra was best demonstrated by a flat differential spectrum. This is consistent with the observed similarities in amino acid composition, but also suggests that the environment of the aromatic residues is very similar.

The peptide maps obtained after tryptic and chymotryptic digestion of the cholecalciferols in the absence of Ca^{2+} are shown in Figs. 7(a) and 7(b). These maps, which clearly differ from those of digests of globin and calmodulin carried out in parallel, are very similar to each other. The tryptic digests are extremely close, down to the presence of a doublet at 27 min in both. The chymotryptic digest patterns are also superimposable. Thus, there is every indication that the peptides produced by chymotryptic and tryptic digestion of the renal and cerebellar cholecalciferols are essentially the same. The inclusion of 1 mM- CaCl_2 in one of the tryptic digests (Fig. 7c) did not

prevent hydrolysis of the cholecalciferols, and may, at most, have slowed the process slightly. This is in sharp contrast with the observations of Drabikowski *et al.* (1977), who showed that the binding of Ca^{2+} to calmodulin caused a conformational change that rendered the molecule resistant to trypsin (Fig. 7c).

The *N*-terminal sequences of both cholecalciferols were identical (Gly-Gly-Val-Ser...), providing further evidence for the close similarity of the two proteins. The presence of a free *N*-terminus sets them apart from the mammalian intestinal cholecalciferols, since all examples of the latter that have been examined to date (Hofmann *et al.*, 1979; Fullmer & Wasserman, 1980) have *N*-terminal residues blocked by acylation.

Thus the parameters of the rat renal and cerebellar cholecalciferols examined in the present study all indicate that the two proteins are so closely related that they may well be identical. The major difference is their vitamin D-dependence in the post-weaning animal, and this could

Table 1. Amino acid compositions of rat renal and cerebellar CaBPs

Source of CaBP...	Rat cerebellar		Rat kidney		Chick intestinal	
Amino acid	Mol %	No. of residues (nearest integer)	Mol %	No. of residues (nearest integer)	Mol %	No. of residues (nearest integer)
Aspartic acid	10.8	27	13.1	32	13.8	31
Threonine†	5.1	13	5.4	13	3.9	9
Serine†	6.7	16	5.9	15	4.3	10
Glutamic acid	15.1	37	15.9	39	17.1	39
Proline	1.4	3	1.5	4	1.3	3
Glycine	9.7	24	9.3	23	5.9	13
Alanine	6.7	16	7.9	20	7.4	17
Valine†	2.1	5	2.2	5	2.2	5
Methionine	3.2	8	1.9	5	2.9	7
Isoleucine	4.2	10	4.2	10	4.7	11
Leucine	12.5	31	12.5	31	12.9	30
Tyrosine	2.9	7	2.6	6	3.4	8
Phenylalanine	5.1	13	4.6	11	5.9	13
Histidine	2.7	7	1.8	4	1.8	4
Lysine	7.8	19	8.1	20	9.9	22
Arginine	3.9	10	2.8	7	2.3	5
Cysteine‡	1.7	4	2.4	6	0.4	1
Tryptophan§	++	++	++	++	0.9	2
M_r		28000		28000		27100

* From Bredderman & Wasserman (1974).

† Compositions were determined after 24, 48 and 72 h of hydrolysis. Values for serine and threonine are zero-time extrapolations. The value for valine is from the 72 h hydrolysate.

‡ Cysteine was determined as carboxymethylcysteine after 24 h hydrolysis.

§ Mercaptoethanol during hydrolysis protects tryptophan somewhat from complete destruction. Although quantification was not attempted, the presence of a tryptophan peak was clear on the chromatograms.

|| ++ indicates the presence of tryptophan.

Fig. 7. Peptide mapping in reverse-phase h.p.l.c.

(a) Tryptic digestion of renal and cerebellar cholecalcins, globin and calmodulin in the absence of Ca^{2+} ; (b) chymotryptic digestion of renal and cerebellar cholecalcins in the absence of Ca^{2+} ; (c) tryptic digestion of renal and cerebellar cholecalcins and calmodulin in the presence of 0.1 mM-Ca^{2+} .

be a function of the specific cells in which they are found rather than of the proteins themselves (Thomasset *et al.*, 1982). The cholecalciferol of cerebellar Purkinje cells is detectable almost as soon as the cells themselves can be identified (embryonic d17) (Legrand *et al.*, 1983). Once these cells are formed and differentiated, they are not replaced during the life of the animal. There is evidence that, although vitamin D can induce cell differentiation (Harmand *et al.*, 1984), it acts only on cells before they become differentiated (Taylor *et al.*, 1984). Therefore any effect on the Purkinje-cell cholecalciferol level would have to occur *in utero*, when the embryo is well buffered against changes in vitamin D.

The results obtained in the present study did not enable us to differentiate between rat cerebellar and renal cholecalciferol by any of the techniques employed. However, the absolute confirmation of the identity of the two proteins will require the determination of their primary structure.

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