Target cell metabolism of 1,25-dihydroxyvitamin D₃ to calcitroic acid

Evidence for a pathway in kidney and bone involving 24-oxidation

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1,25-dihydroxyvitamin D₃ is converted to calcitroic acid before being excreted in the bile. Biosynthesis of calcitroic acid has been demonstrated in two target cells of vitamin D, in the kidney and the osteoblastic cell line UMR-106. Calcitroic acid was identified by combinations of h.p.l.c., u.v. spectroscopy and mass spectrometry. Evidence is presented that calcitroate is derived from the 24-oxidation pathway, possibly through the intermediate 24,25,26,27-tetranor-1,23-dihydroxyvitamin D₃. The 24-oxidation pathway to calcitroic acid in bone cells is stimulated by 1,25-dihydroxyvitamin D₃. The pathway in both bone cells and perfused kidney operates at physiological concentrations of substrate and appears to be capable of rapid clearance of the hormone.

INTRODUCTION

Vitamin D performs a number of physiological functions related to calcium and phosphate homeostasis (DeLuca & Schnoes, 1984). After activation to its hormonal form, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (Fraser & Kodicek, 1970), the molecule is able to execute its actions in a variety of targets including the intestinal mucosal cell, the osteoblast and the renal tubular cell (Price & Baukol, 1980; Nemere *et al.*, 1984; Chandler *et al.*, 1984). The mechanism by which 1,25(OH)₂D₃ acts at target cells involves a specific steroid-hormone-like receptor protein, the gene for which has been recently isolated and cloned (McDonnell *et al.*, 1987). Work performed using rats *in vivo* (Harnden *et al.*, 1976; Esvelt *et al.*, 1979) suggests that the metabolic fate of 1,25(OH)₂D₃, after it has performed its function, is excretion in bile as the C₂₃ acid, calcitroic acid. Since this compound has lost four carbon atoms (C₂₄–C₂₇) present in the side-chain of 1,25(OH)₂D₃, a pathway must exist for the cleavage of the side-chain of vitamin D. To date, the details of this pathway and its cellular location(s) have never been elucidated.

Two pathways have been proposed as possible starting points that might generate calcitroic acid (Napoli & Horst, 1983; Jones, 1986). One pathway begins with 24-hydroxylation and leads to 24-oxo-1,23,25(OH)₃D₃ (Napoli *et al.*, 1983; Mayer *et al.*, 1983b; Jones *et al.*, 1987), while the other begins with 23-hydroxylation and leads to 1,25(OH)₂D₃-26,23-lactone (Napoli & Horst, 1983; Yamada *et al.*, 1984). The demonstrations that the 24-hydroxylation pathway continues further with the formation of a side-chain cleaved molecule, a C₂₃ alcohol

(Jones *et al.*, 1983; Reddy *et al.*, 1987; Lohnes & Jones, 1987) provided a strong indicator that the 24-hydroxylation pathway would turn out to be the source of the intermediates leading to calcitroic acid. In this paper we report the formation of calcitroic acid from 1,25(OH)₂D₃ in two target cell systems *in vitro* and we provide evidence for the role of the 24-hydroxylation pathway in this metabolic process.

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EXPERIMENTAL

Materials

UMR-106 cells (Partridge *et al.*, 1980) were obtained from J.N.M. Heersche, University of Toronto, Toronto, Canada. Trypsin, penicillin G, gentamycin, fungizone and McCoy's 5A medium were purchased from Gibco. Fetal calf serum was from Flow Laboratories. Unlabelled 1,25(OH)₂D₃ and 1,24,25(OH)₃D₃ were generous gifts from Dr. M. Uskokovic of Hoffman–LaRoche. Solvents were of h.p.l.c. grade from Caledon (Georgetown, Ontario, Canada). 24-oxo-1,25(OH)₂D₃, 24-oxo-1,23,25(OH)₃D₃ and 24,25,26,27-tetranor-1,23(OH)₂D₃ were produced from the perfused rat kidney and identified through mass spectrometry (Jones *et al.*, 1987; Lohnes & Jones, 1987).

H.p.l.c.

Automatic analytical h.p.l.c. was performed using a modular instrument comprising a model 510 pump, a

Abbreviations used: 25(OH)D₃, 25-hydroxyvitamin D₃; 24,25(OH)₂D₃, 24(R),25-dihydroxyvitamin D₃; 24-oxo-25(OH)D₃, 24-oxo-25-hydroxyvitamin D₃; 24-oxo-23,25(OH)₂D₃, 24-oxo-23(S),25-dihydroxyvitamin D₃; 24,25,26,27-tetranor-23(OH)D₃, 24,25,26,27-tetranor-23-hydroxyvitamin D₃; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 1,24,25(OH)₃D₃, 1,24(R),25-trihydroxyvitamin D₃; 24-oxo-1,25(OH)₂D₃, 24-oxo-1,25-dihydroxyvitamin D₃; 24-oxo-1,23,25(OH)₃D₃, 24-oxo-1,23(S),25-trihydroxyvitamin D₃; 24,25,26,27-tetranor-1,23(OH)₂D₃, 24,25,26,27-tetranor-1,23-dihydroxyvitamin D₃; 1,25(OH)₂D₃-26,23-lactone, 1,25-dihydroxyvitamin D₃-26,23-lactone.

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WISP automatic sample injector and fixed wavelength u.v. detector (254 nm) (all from Waters Associates, Milford, MA, U.S.A.). Effluent was collected in 30-s fractions using a programmable fraction collector (Superrac; Pharmacia, Dorval, Quebec, Canada) and these were allowed to evaporate to dryness at room temperature in a fume hood, prior to scintillation counting.

Preparative h.p.l.c. involved use of similarly sized columns to those used in analytical analyses, but included substitution of a diode-array detector (model 990; Waters) for the fixed-wavelength detector in order to acquire u.v.-spectral data (200–350 nm) of chromatographic effluent during h.p.l.c. This instrument was used during purification steps to select peaks possessing the u.v. spectral characteristics of the vitamin D *cis*-triene system (u.v._{max.} = 265 nm, u.v._{min.} = 228 nm, $A_{265/228}$ = 1.8–2.0). Fractions were selected and pooled for subsequent steps of purification or alternative methods of identification.

Synthesis of [1β - ^3H]1,25(OH) $_2\text{D}_3$

[1β - ^3H]1,25(OH) $_2\text{D}_3$ was synthesized by the sodium borotritide reduction of 1-oxo,25-hydroxyprevitamin D_3 according to the method of Holick *et al.* (1980). Briefly, 3 mg of 1-oxo precursor, dissolved in 200 μl of methanol/10 μl of water, was reduced with 3 mg of sodium borotritide (5 Ci; 80 Ci/mmol) at 0 °C for 75 min using commercially available tritium-labelling services (Amersham) to give a mixture of [1β - ^3H]1 α ,25-dihydroxyprevitamin D_3 and [1α - ^3H]1 β ,25-dihydroxyprevitamin D_3 in a ratio of 1:10. The two isomers were resolved by h.p.l.c. on a 25 cm $\times$ 4.6 cm Resolve 5 μm particle size column (Waters, Milford, MA, U.S.A.) using the solvent system hexane/propan-2-ol/methanol (94:5:1, v/v) at a flow rate of 2 ml/min. [1α - ^3H]1 β ,25-dihydroxyprevitamin D_3 emerged at 6 min and [1β - ^3H]1 α ,25-dihydroxyprevitamin D_3 at 9 min using this h.p.l.c. system. The thermal product of [1β - ^3H]1 α ,25-dihydroxyprevitamin D_3 {[1β - ^3H]1 α ,25(OH) $_2\text{D}_3$; 20 Ci/mmol} had a u.v. spectrum with u.v._{max.} = 265 nm; u.v._{min.} = 228 nm, and co-migrated with authentic crystalline 1 α ,25(OH) $_2\text{D}_3$ on two chromatographic systems (Zorbax-Sil; Jones, 1980; Zorbax-CN; Jones, 1983). The pure compound was stored in ethanol at –30 °C until used.

Perfused rat kidney

Rats (250–300 g) were fed a standard rodent chow (Purina). In order to maximize the activity of side-chain hydroxylases, animals were injected intraperitoneally on the day before sacrifice with 1,25(OH) $_2\text{D}_3$ (1.5 ng/g of body wt.) dissolved in plasma from vitamin D-deficient rats. Kidneys were isolated and perfused as described previously (Rosenthal *et al.*, 1980; Reddy *et al.*, 1982). 1 α ,25(OH) $_2\text{D}_3$ (containing 1β - ^3H -labelled and/or non-radioactive forms as specified in the Results section) was added to the venous reservoir dissolved in 100 μl of ethanol. Perfusion lasted a duration of 6 h, and 4 ml aliquots of perfusate were removed every hour. Those aliquots and the perfusate remaining at the end of the experiment, which averaged 100 ml in volume, were stored at –30 °C until extraction.

Bone cell culture

UMR-106 cells were seeded at an initial density of 20 000 cells/cm 2 in McCoy's modified 5A medium con-

taining 10% fetal calf serum and antibiotics (100 μg of penicillin G/ml, 5 μg of gentamycin/ml and 300 ng of fungizone/ml). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO $_2$ in air. Cultures were routinely grown to late log phase before use. In the experiments described below, 1,25(OH) $_2\text{D}_3$ was added in ethanolic solution, the ethanol concentration not exceeding 0.1% (v/v).

In time course analyses, cells were cultured in 2 cm 2 multiwell plates. Monolayers were washed with 1 ml of Dulbecco's modified salt solution and then incubated for 4 h with 10 nM-1,25(OH) $_2\text{D}_3$ in McCoy's medium containing 10% fetal calf serum to induce side-chain hydroxylase enzymes. Following the induction period, cultures were washed with Dulbecco's modified salt solution (2 $\times$ 1 ml washes) and incubated with 5 nM-[1β - ^3H]1,25(OH) $_2\text{D}_3$ (specific activity 20 Ci/mmol) in 500 μl of medium containing 10% fetal calf serum for 0–4 h. Cell numbers were assessed in parallel cultures using a Coulter counter (model ZM; Coulter Electronics, Luton, Beds., U.K.).

In studies designed to generate [1β - ^3H]calcitric acid, cells (1.5×10^7) were grown to late log phase in 75 cm 2 flasks, washed with 5 ml of Dulbecco's modified salt solution and incubated with 15 nM-[1β - ^3H]1,25(OH) $_2\text{D}_3$ (7 Ci/mmol) for 30 h in 10 ml of McCoy's medium containing 10% fetal calf serum.

In preparative studies aimed at generating calcitric acid for diode-array spectrophotometry, thirty plates of cells were each cultured to late log phase (30×10^6 cells/plate) in 150 mm diam. culture dishes and then incubated for a further 5 days in the presence of 10 μg of 1,25(OH) $_2\text{D}_3$ (1.55 μM) in 15 ml of medium containing 10% fetal calf serum.

Incubations of bone cells with 1,25(OH) $_2\text{D}_3$, both large and small scale, were terminated by the addition of methanol, which constituted the first step of lipid extraction. No attempt was made to separate bone cells from medium in these studies.

Lipid extraction

For analytical scale studies using aliquots of perfusate or small numbers of bone cells, lipid extraction was carried out in 30 ml test tubes using a modification of the method of Bligh & Dyer (Bligh & Dyer, 1957) substituting methylene chloride for chloroform and substituting distilled water for saturated KCl.

For extraction of final renal perfusates (volume 100 ml) of preparative scale bone cell cultures (volume 600 ml), the same procedure was carried out using 2 l separatory funnels instead of 30 ml test tubes.

In the case of both analytical and preparative scale samples, the methylene chloride layer, containing unchanged 1,25(OH) $_2\text{D}_3$ and lipid-soluble metabolites, was evaporated to dryness and the residue redissolved in hexane/propan-2-ol/methanol (88:10:2, by vol.) for h.p.l.c. analysis. The fate of the upper aqueous layer from Bligh & Dyer extractions depended on the scale used and is described in the following sections.

Chromatography of ^3H -labelled aqueous soluble metabolites

The aqueous layer from lipid extraction of renal perfusates, containing putative calcitric acid and other polar vitamin D metabolites, was diluted 10-fold with deionized water in order to reduce the methanol con-

centration to less than 5%. C₁₈ Sep-Pak cartridges (Waters) were washed with 2 ml of acetonitrile and 5 ml of water prior to use. The diluted aqueous layer was then applied to a prewashed C₁₈ Sep-Pak cartridge. Suction generated by a vacuum pump was used to draw the diluted aqueous layer through the cartridge mounted in a Sep-Pak apparatus (Waters). After application of the diluted aqueous layer, the Sep-Pak was washed with 20 ml of distilled water in order to elute loosely bound water-soluble compounds and polar lipids. Calcitroic acid and other polar vitamin D metabolites were then eluted with 15 ml of acetonitrile containing 0.1% glacial acetic acid. This fraction was evaporated to dryness on a rotary evaporator under vacuum, redissolved in 3 ml of acetonitrile, transferred to a 5 ml Reactivial (Pierce) and evaporated to dryness under N₂. The residue was redissolved in 200 μ l of acetonitrile/water (40:60, v/v) and subjected to reverse-phase h.p.l.c. on Zorbax-ODS (4.6 mm $\times$ 25 cm) (Dupont) using acetonitrile/water (40:60, v/v) as mobile phase at 2 ml/min. Polar metabolites of [$^{1-3}$ H]1,25(OH)₂D₃ were detected in effluent by collection of 0.5 min fractions (1 ml) and mixing with aqueous scintillation fluid (Readysolve HP; Beckman) followed by liquid scintillation counting.

Preparative scale purification of [3 H]calcitroic acid

For generation of sufficient quantities of the putative calcitroic acid peak from kidney, we took the aqueous layer from 100 ml of perfusate and diluted it 10-fold as above. Portions (8 $\times$ 600 ml) of diluted aqueous layer were then fractionated on eight C₁₈ Sep-Paks as described above to give a single pooled fraction containing polar metabolites of 1,25(OH)₂D₃. The fraction was rotary evaporated to dryness, the residue redissolved in acetonitrile and transferred to a Reactivial for further sample concentration. After evaporation under a stream of N₂, the residue was redissolved in 2.1 ml of acetonitrile/water (40:60, v/v). Ten 200 μ l aliquots of the extract were subjected to reverse-phase h.p.l.c. on Zorbax-ODS as above. Fractions of effluent (40 $\times$ 0.5 min) were collected from each run and subsequently pooled to give 40 fractions representing 0 to 0.5 min, 0.5 to 1 min, etc. A single 100 μ l aliquot of the extract was chromatographed as above and collected into 40 $\times$ 0.5 min fractions for liquid scintillation counting. The chromatographic profile obtained from these fractions revealed that the putative calcitroic acid peak emerged at around 7.5 min (see Table 1, System 1). Fractions 12–16 from the pooled 10 runs were combined to represent the calcitroic acid fraction. The peak was diluted with ethanol, rotary evaporated to dryness and the residue redissolved in 100 μ l of methanol for methylation.

The isolation of the putative calcitroic acid peak from the aqueous layer of bone cells (10⁹) was slightly modified. Instead of using Sep-Pak cartridges, we simply acidified the bulk aqueous layer with 5% glacial acetic acid and re-extracted with a second portion of methylene chloride. The methylene chloride was evaporated to dryness and the residue redissolved in acetonitrile/glacial acetic acid (99:1, v/v). Subsequent steps of purification were the same as with the renal metabolite. However, unlike the renal calcitroic acid, the bone calcitroic acid was not methylated and mass spectrometry was not performed on it.

Methylation of putative calcitroic acid

Diazomethane was generated by dripping 0.6 ml of NaOH (5 M) on to 100 mg of 1-methyl-3-nitro-1-nitroso-guanidine (MNNG) inside the sealed inner chamber of a double-chambered MNNG–Diazomethane Generation Apparatus (all from Aldrich Chemicals, Milwaukee, WI, U.S.A.). Ethereal solution of diazomethane (1 ml) was added to 100 μ l of methanol containing the putative calcitroic acid and the product (250 ng) purified by h.p.l.c. for mass spectrometry. Methyl calcitroate was collected as a single peak at 9.7 min from a Zorbax-Sil, 3 μ m particle size column using the mobile phase hexane/propan-2-ol/methanol (91:7:2, by vol.) at a flow rate of 1 ml/min (Lohnes & Jones, 1987).

Mass spectrometry

Putative methyl calcitroate from kidney preparations was subjected to electron-impact-mass spectrometry using a model HP5985 mass spectrometer (Hewlett–Packard) fitted with a direct insert probe as described previously (Lohnes & Jones, 1987). Samples (250 ng) were introduced into glass capillary inserts in 20 μ l of hexane/propan-2-ol/methanol (94:5:1, by vol.) and evaporated to dryness under vacuum just prior to mass spectral analysis.

RESULTS

Renal metabolism of 1,25(OH)₂D₃

The pattern of metabolism of [$^{1-3}$ H]1,25(OH)₂D₃ by the isolated perfused rat kidney varied as the concentration of substrate was increased from 400 pM to 1 μ M (Figs. 1a, 1b and 1c). The rise in lipid-soluble metabolites of 1,25(OH)₂D₃, defined as those metabolites recovered from the methylene chloride layer during lipid extraction, did not account for the decline in substrate radioactivity, particularly at substrate concentrations of 400 pM and 200 nM. In fact, so little radioactivity remained in the methylene chloride layer at all time points of perfusion using 400 pM or 200 nM substrate that it was difficult to positively identify any metabolite of 1,25(OH)₂D₃ except 24,25,26,27-tetranor-1,23(OH)₂D₃ (Figs. 1a and 1b). This was achieved by co-migration with authentic standard on a single h.p.l.c. system. Analysis of the lipid-soluble metabolites from a 6 h perfusion using 1 μ M substrate (Fig. 1c) revealed the presence of a number of 3 H-metabolites corresponding to compounds identified previously (Napoli *et al.*, 1983; Mayer *et al.*, 1983b; Jones *et al.*, 1987; Lohnes & Jones, 1987). U.v. analysis of the same lipid-soluble metabolites from a 6 h kidney perfusion using 1 μ M-1,25(OH)₂D₃ gave peaks that were visible on h.p.l.c. by their vitamin D chromophore (results not shown) as well as their 3 H label. Peaks made in the perfused kidney which possessed the vitamin D chromophore and the 1- 3 H label, arranged in the expected order of appearance in their biosynthetic pathway, were: 1,24,25(OH)₃D₃, 24-oxo-1,25(OH)₂D₃, 24-oxo-1,23,25(OH)₃D₃ and 24,25,26,27-tetranor-1,23(OH)₂D₃. With higher substrate concentrations and as the time of incubation increased, there was a progressive switch from accumulation of tetranor-1,23(OH)₂D₃ to a predominance of the earlier metabolites in the pathway.

We investigated the time course of appearance of water-soluble radioactivity from the metabolism of [$^{1-3}$ H]1,25(OH)₂D₃ in the isolated perfused kidney at both

Fig. 1. H.p.l.c. of the organic layer from perfusate extractions from rat kidneys perfused with (a) 400 pM-, (b) 200 nM- or (c) 1 μ M-[1- 3 H]1,25(OH) $_2$ D $_3$.

H.p.l.c. conditions for all were: Zorbax-SIL 3 μ m particle size, hexane/propan-2-ol/methanol (88:10:2, by vol.), flow rate 1 ml/min. Within each figure, profiles represent perfusate removed at 2 h (—), 4 h (----) and 6 h (---). Numbers above peaks represent positions of standards: 1, 1,25(OH) $_2$ D $_3$; 2, 24-oxo-1,25(OH) $_2$ D $_3$; 3, tetranor-1,23(OH) $_2$ D $_3$; 4, 24-oxo-1,23,25(OH) $_3$ D $_3$; 5, 1,24,25(OH) $_3$ D $_3$. Note the sharp decline in substrate (peak 1) radioactivity at 400 pM substrate concentration, with smaller declines at higher substrate concentrations.

the 200 nM and 1 μ M substrate concentrations (results not shown). There was a significant linear time-dependent increase in water-soluble radioactivity, defined as the radioactivity present in the upper aqueous layer during lipid extraction. At a concentration of 200 nM-1,25(OH) $_2$ D $_3$, 50.8% ($n=2$; values 49.5 and 52.2) of the substrate had been converted to water-soluble metabolites after 6 h of perfusion. In contrast, at a

Fig. 2. H.p.l.c. of the aqueous layer from the lipid extraction of renal perfusate

Perfusion was carried out for 6 h at 1 μ M-[1- 3 H]1,25(OH) $_2$ D $_3$. Conditions for h.p.l.c. were Zorbax-ODS, 3 μ m particle size, acetonitrile/water (40:60, v/v), 2 ml/min; the detector was a diode-array spectrophotometer. ---, Radioactivity profile (0.5 min fractions); —, ultraviolet absorbance at 265 nm.

concentration of 1 μ M substrate, only $20 \pm 2.8\%$ ($n=4$, mean $\pm$ S.D.) of the starting material was converted to water-soluble metabolites after 6 h. The increase in water-soluble radioactivity with time was linear over a 6 h period with a slope of 1.95 nmol/h per g of tissue at 200 nM substrate and 3.94 nmol/h per g of tissue at 1 μ M-substrate. Total recovered radioactivity calculated by summing lipid-soluble and water-soluble radioactivity remained stable at all time points, demonstrating that the 1- 3 H label was not lost to volatile products during extraction.

Reverse-phase h.p.l.c. analysis of the aqueous layer from lipid extraction revealed the existence of four peaks representing 1- 3 H-labelled polar metabolites of 1,25(OH) $_2$ D $_3$ (Fig. 2). One of these contained considerable amounts of radioactivity but was at the void volume of the column. The main metabolite peak running at 7.2 min (Fig. 2) and in fraction 15 of the radioactivity profile was putative calcitroic acid. Two other minor peaks were unidentified, but one peak co-migrated with tetranor-1,23(OH) $_2$ D $_3$ and thus probably represents small amounts of this metabolite partitioning into the aqueous layer.

Identification of calcitroic acid from the perfused kidney

The putative calcitroic acid peak from Zorbax-ODS h.p.l.c. showed a distinctive pattern of chromatographic behaviour on several h.p.l.c. systems (Table 1). The metabolite did not co-migrate with any of the known side-chain-modified, non-polar compounds that were run as standards. However, since synthetic standard calcitroic acid was unavailable, co-chromatography was not feasible. As can be seen by comparing the retention times of the putative calcitroic acid using systems 3 and 4 in Table 1, the metabolite was found to be sensitive to acidic conditions, emerging >8 min earlier in the presence of 1% glacial acetic acid than in its absence. This indicates the presence of a polar functionality in the metabolite, probably a carboxylic acid substitution.

Both the native and methylated metabolite retained the distinctive chromophore of vitamin D, indicating no change in the *cis*-triene region of the molecule. The

Renal calcitroic acid represents the material generated in the perfused kidney and subsequently methylated and subjected to mass spectrometry. System 1, Zorbax-ODS 3 μm particle size, acetonitrile/water (40:60, v/v), 2 ml/min; system 2, Zorbax-ODS 3 μm , acetonitrile/water/glacial acetic acid (40:60:1, by vol.), 2 ml/min; system 3, Zorbax-Sil 3 μm , hexane/propan-2-ol/methanol (88:10:2, by vol.), 2 ml/min; system 4, Zorbax-Sil 3 μm , hexane/propan-2-ol/methanol/glacial acetic acid (88:10:2:1, by vol.), 2 ml/min; system 5, Zorbax-Sil 3 μm , hexane/propan-2-ol/methanol (76:20:4; by vol.), 2 ml/min. N.D., not determined.

	Retention time (min)				
	System 1	System 2	System 3	System 4	System 5
Calcitroic acid	7.3	4.69	> 12	4.70	6.42
Methyl calcitroate	N.D.	N.D.	3.70	N.D.	1.70
1,25(OH) ₂ D ₃	65.0	> 60.0	4.80	4.53	1.95
24-oxo-1,25(OH) ₂ D ₃	23.5	17.4	5.45	5.55	N.D.
tetranor-1,23(OH) ₂ D ₃	12.1	6.6	6.60	6.33	2.30
24-oxo-1,23,25(OH) ₃ D ₃	8.9	6.1	7.35	7.00	2.23
1,24,25(OH) ₃ D ₃	15.7	8.3	8.40	7.60	2.55

change in retention time after methylation to that of a non-polar vitamin D again suggested the presence of a carboxylic acid group.

Mass spectrometry of the native metabolite proved to be unsuccessful, presumably due to its charge. The methyl ester of the kidney metabolite gave a distinctive mass spectrum (Fig. 3) with a molecular ion at m/z 388 and several characteristic fragments present in the original mass spectrum published by Esvelt *et al.* (1979). The mass spectrum shows unique ions at m/z 370 ($M^+ - \text{H}_2\text{O}$), 352 ($370 - \text{H}_2\text{O}$), 339 ($M^+ - \text{OCH}_3$ and H_2O). In addition, the mass spectrum exhibits ions at m/z 287 (M^+ -side chain), 269 ($287 - \text{H}_2\text{O}$), 251 ($269 - \text{H}_2\text{O}$), 152 (*cis*-triene cleavage) and 134 ($152 - \text{H}_2\text{O}$), all of which are present in the spectrum of $1,25(\text{OH})_2\text{D}_3$ and its 1-hydroxylated derivatives and which are indicative of a vitamin D with an intact *cis*-triene and a 1-hydroxylated A-ring. In summary, all the mass spectral data are consistent with a 1-hydroxylated vitamin D with a side chain truncated at C_{23} and containing a carboxylic acid group. On the basis of mass and u.v. spectral data as well as h.p.l.c. data presented, we conclude that the metabolite is calcitric acid.

Using $[1\beta\text{-}^3\text{H}]24\text{-oxo-}1,23,25(\text{OH})_3\text{D}_3$ and $[1\beta\text{-}^3\text{H}]$ -tetranor- $1,23(\text{OH})_2\text{D}_3$ isolated from the lipid-soluble fraction of renal perfusates, we set out to establish the nature of the intermediates involved in the synthesis of calcitroic acid. Kidneys from vitamin D-replete animals were perfused in the presence of either radioactive compound, and reverse-phase h.p.l.c. was performed on aliquots of perfusate. Aliquots of perfusate removed at 2, 4 or 6 h revealed a time-dependent synthesis of calcitroic acid from either precursor (results not shown). The $[1\beta\text{-}^3\text{H}]$ calcitroic acid peak formed from both $1\beta\text{-}^3\text{H}$ -labelled intermediates co-migrated with the putative calcitroic acid on two h.p.l.c. systems (systems 2 and 4 of Table 1). Calcitroic acid accounted for between 13 and 23% of recovered radioactivity at 6 h of incubation. The use of $[1\beta\text{-}^3\text{H}]24\text{-oxo-}1,23,25(\text{OH})_3\text{D}_3$ as substrate also led to the synthesis of $[1\beta\text{-}^3\text{H}]$ tetranor- $1,23(\text{OH})_2\text{D}_3$ in addition to production of $[1\beta\text{-}^3\text{H}]$ calcitroic acid, confirming earlier suggestions of the order of the metabolites in this pathway (Jones *et al.*, 1983). As with perfusions involving $[1\beta\text{-}^3\text{H}]1,25(\text{OH})_2\text{D}_3$, $[1\beta\text{-}^3\text{H}]24\text{-oxo-}1,23,25(\text{OH})_3\text{D}_3$

Fig. 4. H.p.l.c. of extracts of bone cells incubated with [1β - ^3H]1,25(OH) $_2\text{D}_3$.

(a) Methylene chloride layer on Zorbax-Sil, 3 μm particle size, hexane/propan-2-ol/methanol (91:7:2, by vol.), flow rate 2 ml/min (Lohnes & Jones, 1987). (b) Aqueous layer on Zorbax-ODS, 3 μm ; acetonitrile/water/glacial acetic acid (40:60:1, by vol.), 2 ml/min. Peaks numbers represent tentative assignment based upon a single h.p.l.c. run by comparison with retention time of standards. 1, 1,25(OH) $_2\text{D}_3$; 2, 24-oxo-1,25(OH) $_2\text{D}_3$; 3, tetranor-1,23(OH) $_2\text{D}_3$; 4, 24-oxo-1,23,25(OH) $_3\text{D}_3$; 5, 1,24,25(OH) $_3\text{D}_3$; 6, unknown; 7, unknown; 8, calcitroic acid; 9, tetranor-1,23(OH) $_2\text{D}_3$; 10, 1,24,25(OH) $_3\text{D}_3$.

and tetranor-1,23(OH) $_2\text{D}_3$ also yielded considerable amounts of radioactivity which ran in the void volume of the Zorbax-ODS column, possibly representing highly polar unidentified metabolites. In summary, the evidence obtained thus far suggests that the renal calcitroic acid is derived from 24-oxo-1,23,25(OH) $_3\text{D}_3$, possibly through the intermediate 24,25,26,27-tetranor-1,23(OH) $_2\text{D}_3$.

1,25-(OH) $_2\text{D}_3$ metabolism in bone cells

Metabolism of [1β - ^3H]1,25(OH) $_2\text{D}_3$ by the bone cell line UMR-106 was dependent upon the prior treatment of the cells with 10 nM-1,25(OH) $_2\text{D}_3$. As shown in Fig. 4(a), cells grown in the presence of 10 nM-1,25(OH) $_2\text{D}_3$ for 4 h and then switched to growth medium containing 5 nM-[1β - ^3H]1,25(OH) $_2\text{D}_3$ were able to synthesize several known ^3H -labelled metabolites (Lohnes & Jones, 1987), the principle one being [1β - ^3H]24,25,26,27-tetranor-1,23(OH) $_2\text{D}_3$. As with the kidney metabolism of physiological concentrations of [1β - ^3H]1,25-(OH) $_2\text{D}_3$, the labelled compound was rapidly converted to tetranor-

Fig. 5. U.v. spectra of renal and bone cell calcitroic acid during h.p.l.c.

Chromatographic conditions: (a) Zorbax-Sil, 3 μm particle size, hexane/propan-2-ol/methanol, 76:20:4, by vol., flow rate 2 ml/min; (b) Zorbax-ODS, 3 μm , acetonitrile/water/glacial acetic acid (40:60:1, by vol.), 2 ml/min. Note that wavelengths 210–230 nm are omitted because these are blocked out by glacial acetic acid in the solvent. In both (a) and (b), bone calcitroic acid is indicated by the solid line and kidney calcitroic acid by the dashed line. All spectra were collected at the apex of h.p.l.c. peaks. Retention times are provided in the text.

1,23(OH) $_2\text{D}_3$ without appreciable accumulation of side-chain-modified intermediates. It also became evident from these analyses of the organic layer that considerable amounts of radioactivity were being converted into water-soluble products. Time-course analyses over a 4 h period revealed that, as with the kidney, lipid-soluble radioactivity was replaced by water-soluble radioactivity. The production of water-soluble radioactivity was linear over a 4 h incubation period with a slope of 205 fmol/h per 10^6 cells at a 1,25(OH) $_2\text{D}_3$ concentration of 10 nM (results not shown).

Analysis of the radioactivity in the aqueous layer again showed the presence of several water-soluble metabolites, including a peak of radioactivity resembling the putative calcitroic acid on reverse-phase h.p.l.c. (Fig. 4b).

Table 2. Comparison of calcitroic acid production from 1,25(OH)₂D₃ and tetranor-1,23(OH)₂D₃ in cultures of UMR-106

Cells were treated for 4 h with 10 nmol of 1,25(OH)₂D₃ or ethanol. The medium was changed and cells were incubated a further 2 h in the presence of [³H]1,25(OH)₂D₃ or [³H]tetranor-1,23(OH)₂D₃ (1.2 nM final concentration). Calcitroic acid was separated using the h.p.l.c. system: Zorbax-ODS, 3 µm particle size, acetonitrile/water/glacial acetic acid (40:60:1, by vol.), 2 ml/min, and quantified by liquid scintillation counting. Results represent means ± S.D. from triplicate determinations. N.S., not significantly different from no-cell controls.

Substrate	Calcitroic acid synthesis (fmol/h per 10 ⁶ cells)	
	Ethanol- pre-treated cells	1,25(OH) ₂ D ₃ - pre-treated cells
1,25(OH) ₂ D ₃	N.S.	37.9 ± 1.8
tetranor- 1,23(OH) ₂ D ₃	N.S.	35.2 ± 1.1

Fig. 6. Proposed pathway from 1,25(OH)₂D₃ to calcitroic acid
SCC, side-chain cleavage enzyme.

Identification of bone cell calcitroic acid

Preparative studies involving incubation of 300 µg of 1,25(OH)₂D₃ with 10⁹ cells for 5 days gave a low yield of the putative bone calcitroic acid peak (200 ng). However, the quantity of metabolite generated did permit the use of diode-array spectrophotometry. Though the metabolite was contaminated by lipid, it gave the characteristic u.v. spectrum of vitamin D on both straight-phase and reverse-phase h.p.l.c. (Fig. 5). In addition, the putative bone-cell calcitroate co-migrated with renal calcitroate on three h.p.l.c. systems (system 2 in Table 1, but flow rates 1 ml/min; 10.80 and 10.85 min respectively; system 4 in Table 1, 4.98 and 4.96 min respectively; and system 5 in Table 1, 5.55 and 5.49 min respectively). Based upon the chromatographic and spectral properties of the bone cell metabolite, we conclude that it is calcitroic acid.

Origin of bone cell calcitroic acid

[1β-³H]tetranor-1,23(OH)₂D₃ (20 Ci/mmol) isolated from the organic layer of bone cell incubates, and the original substrate [1β-³H]1,25(OH)₂D₃ were used to compare the biosynthesis of [1β-³H]calcitroic acid from either substrate in UMR-106 incubates over a 2 h period. As

shown in Table 2, prior treatment of the bone cells with 1,25(OH)₂D₃ was essential in order to demonstrate production of [1β-³H]calcitroic acid using either radio-labelled precursor. Production of calcitroic acid, which routinely represented 5–10 % of recovered radioactivity, was comparable using either 1,25(OH)₂D₃ or tetranor-1,23(OH)₂D₃ (Table 2).

DISCUSSION

In this paper we have presented evidence for the generation *in vitro* of calcitroic acid from 1,25(OH)₂D₃ in two vitamin D targets, the kidney and the osteoblastic cell line UMR-106. This is the first time that the work *in vivo* of Esvelt and co-workers (Esvelt *et al.*, 1979; Esvelt & DeLuca, 1981), suggesting that calcitroic acid is the major excretory metabolite of 1,25(OH)₂D₃, has been confirmed, and it represents the first time that calcitroic acid has been generated *in vitro*. Data presented here show that calcitroic acid is probably derived from the 24-oxidation pathway (Jones, 1986) (Fig. 6) which begins with 1,24,25(OH)₃D₃ and involves a C₂₃–C₂₄ cleavage of 24-oxo-1,23,25(OH)₃D₃ (Jones *et al.*, 1987; Reddy *et al.*, 1987; Lohnes & Jones, 1987). Using 1β-³H-labelled 24-oxidation intermediates we were able to demonstrate both in the perfused kidney and the bone cell line UMR-106 that compounds on the 24-oxidation pathway will act as precursors to [1β-³H]calcitroic acid in a time-dependent manner. Our results do not exclude the possibility that calcitroic acid can be derived from other pathways, for instance the 1,25(OH)₂D₃-26,23-lactone pathway. However, our ability to detect all of the C₂₄-oxidation intermediates in two biological systems making calcitroic acid *in vitro* and our inability to detect 1,25(OH)₂D₃-26,23-lactone and its precursors in the same systems suggest that the 24-oxidative pathway and not the 26,23-lactone pathway is the source of calcitroic acid.

In their initial studies, Esvelt *et al.* (1979) identified calcitroic acid in the bile of rats given [3α-³H]-1,25(OH)₂D₃, and in later studies (Esvelt & DeLuca, 1981) went on to document the tissue distribution of calcitroic acid in the rat. These latter studies (Esvelt & DeLuca, 1981) revealed that many tissues contained measurable amounts of calcitroic acid, but the authors were unable to pinpoint its site of synthesis or elucidate the nature of the intermediates involved in calcitroate biosynthesis. Our work demonstrates that at least two vitamin D target tissues, kidney and bone, possess the ability to synthesize calcitroic acid and that the 24-oxidation pathway probably provides the intermediates leading to calcitroate. Our work also indicates that the enzymes involved in all the steps from 1,25(OH)₂D₃ to calcitroic acid in UMR-106 are 1,25(OH)₂D₃-inducible, requiring a 4 h induction period (Table 2; D. Lohnes & G. Jones, unpublished work). As with our recent studies with the side-chain hydroxylases in UMR-106 (Lohnes & Jones, 1987), the 4 h induction period is highly suggestive of a requirement for a step of protein synthesis under the transcriptional control of 1,25(OH)₂D₃ (Haussler, 1986). Like Esvelt (Esvelt & DeLuca, 1981) we also detected other unidentified polar metabolites of 1,25(OH)₂D₃ which could be alternative products of catabolism or conjugates of calcitroate.

The technical approach used for the study of calcitroic acid in this paper is quite different from that used by Esvelt (Esvelt *et al.*, 1979; Esvelt & DeLuca, 1981) and

deserves some comment. The work of Esvelt *in vivo* required 450 g of rat liver to generate sufficient quantities of calcitroic acid for identification. In our studies we employed models *in vitro* developed in our laboratory and used successfully in previous metabolic studies (Jones, 1986; Lohnes & Jones, 1987). These intact cell models permitted the unambiguous demonstration that kidney and bone could be sources of calcitroic acid *in vivo* while permitting the build-up of large amounts of vitamin D metabolites. We avoided use of broken cell systems *in vitro* such as the renal homogenate because of uncertainty about the subcellular location of the enzymes involved and the possibility that the rate of synthesis of calcitroate might be undetectable in a model with limited viability. In Esvelt's work, both their extraction and purification procedures involved several steps, the latter including ion-exchange and h.p.l.c. In the present studies, we used only two kidneys (approx. 2.2 g of tissue) to generate sufficient calcitroic acid and incorporated a novel procedure using C₁₈ Sep-Pak cartridges during initial stages of purification as well as new procedures for h.p.l.c. By keeping the methodology simple we were able to speed up the isolation of calcitroic acid considerably. Future studies aimed at understanding the regulation of calcitroic acid production should benefit from some of the extraction and h.p.l.c. procedures described here.

Our results suggest that the production of calcitroic acid in target cells for 1,25(OH)₂D₃ is a highly efficient process occurring even at low concentrations of 1,25(OH)₂D₃. This supports the concept put forward by Haussler (1986) and further extended by Lohnes & Jones (1987) that 1,25(OH)₂D₃ at physiological concentrations up-regulates its own receptor in target cells, induces a series of side-chain metabolizing enzymes and thus triggers its own destruction. It is not yet clear whether calcitroic acid crosses target cell membranes but in these studies calcitroic acid was found in the renal perfusate implying that such a mechanism exists. The work of Esvelt *et al.* (1979) suggests that calcitroate is transported *in vivo* in a free or conjugated form to the liver for excretion.

The demonstration of the enzymic formation of calcitroic acid from 1,25(OH)₂D₃ through 1,24,25(OH)₂D₃ in our studies evokes the question, what regulates this pathway *in vivo*? Previous studies have focused on whether 1,24,25(OH)₂D₃ has biological activity *in vivo* (Holick *et al.*, 1973; Mayer *et al.*, 1983a). If instead we view 1,24,25(OH)₂D₃ as a catabolite, we could postulate that its synthesis may be reduced or accelerated in order to attenuate or extend the biological activity of 1,25(OH)₂D₃. 24-Difluoro-1,25(OH)₂D₃ has been reported (Tanaka *et al.*, 1980) to have an increased half-life *in vivo*, perhaps due to the block in 24-oxidation of its side chain. It is also possible that certain pathological conditions could be due to excessive or reduced ability to synthesize calcitroic acid. In this regard, our recent demonstration of increased 24-oxidation of both 25-hydroxyvitamin D [25(OH)D₃] and 1,25(OH)₂D₃ in renal mitochondria from *Hyp* mice compared with their normal littermates is interesting (Tenenhouse *et al.*, 1988). In light of our new data presented here, it will be important to see which step of the pathway to calcitroic acid is regulated by factors other than 1,25(OH)₂D₃ and whether regulation of these steps is important in human disease states.

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REFERENCES

- Bligh, E. G. & Dyer, W. J. (1957) *Can. J. Biochem.* **37**, 911–917
- Chandler, J. S., Chandler, S. K., Pike, J. W. & Haussler, M. R. (1984) *J. Biol. Chem.* **259**, 2214–2222
- DeLuca, H. F. & Schnoes, H. K. (1984) *Annu. Rep. Med. Chem.* **19**, 179–190
- Esvelt, R. P. & DeLuca, H. F. (1981) *Arch. Biochem. Biophys.* **206**, 403–413
- Esvelt, R. P., Schnoes, H. K. & DeLuca, H. F. (1979) *Biochemistry* **18**, 3977–3983
- Fraser, D. R. & Kodicek, E. (1970) *Nature (London)* **228**, 764–766
- Harnden, D., Kumar, R., Holick, M. F. & DeLuca, H. F. (1976) *Science* **193**, 493–494
- Haussler, M. R. (1986) *Annu. Rev. Nutr.* **6**, 527–562
- Holick, M. F., Kleiner-Bossaller, A., Schnoes, H. K., Kasten, P. M., Boyle, I. T. & DeLuca, H. F. (1973) *J. Biol. Chem.* **248**, 6691–6696
- Holick, S. A., Holick, M. F. & MacLaughlin, J. A. (1980) *Biochem. Biophys. Res. Commun.* **97**, 1031–1037
- Jones, G. (1980) *J. Chromatogr.* **221**, 27–37
- Jones, G. (1983) *J. Chromatogr.* **276**, 69–75
- Jones, G. (1986) *Methods Enzymol.* **123**, 141–154
- Jones, G., Kung, M. & Kano, K. (1983) *J. Biol. Chem.* **258**, 12920–12928
- Jones, G., Vriezen, D., Lohnes, D., Palda, V. & Edwards, N. S. (1987) *Steroids* **49**, 29–55
- Lohnes, D. & Jones, G. (1987) *J. Biol. Chem.* **262**, 14393–14401
- Mayer, E., Bishop, J. E., Chandraratna, R. A. S., Okamura, W. H., Kruse, J. R., Popjak, G., Ohnuma, N. & Norman, A. W. (1983a) *J. Biol. Chem.* **258**, 13458–13465
- Mayer, E., Bishop, J. E., Ohnuma, N. & Norman, A. W. (1983b) *Arch. Biochem. Biophys.* **224**, 671–676
- McDonnell, D. P., Mangelsdorf, D. J., Pike, J. W., Haussler, M. R. & O'Malley, B. W. (1987) *Science* **235**, 1214–1217
- Napoli, J. L. & Horst, R. L. (1983) *Biochemistry* **22**, 5848–5853
- Napoli, J. L., Pramanik, B. C., Royal, P. M., Reinhardt, T. A. & Horst, R. L. (1983) *J. Biol. Chem.* **258**, 9100–9107
- Nemere, I., Yoshimoto, Y. & Norman, A. W. (1984) *Endocrinology (Baltimore)* **115**, 1476–1483
- Partridge, N. C., Frampton, R. J., Eisman, J. A., Michelangeli, V. P., Elms, E., Bradley, T. R. & Martin, T. J. (1980) *FEBS Lett.* **115**, 139–142
- Price, P. A. & Baukol, S. A. (1980) *J. Biol. Chem.* **255**, 11660–11663
- Reddy, G. S., Jones, G., Kooh, S. W. & Fraser, D. (1982) *Am. J. Physiol.* **243**, E265–E271
- Reddy, G. S., Tserng, K., Thomas, B. R., Dayal, R. & Norman, A. W. (1987) *Biochemistry* **26**, 324–330
- Rosenthal, A. M., Jones, G., Kooh, S. W. & Fraser, D. (1980) *Am. J. Physiol.* **239**, E12–E20
- Tanaka, Y., DeLuca, H. F., Schnoes, H. K., Ikekawa, N. & Kobayashi, Y. (1980) *Arch. Biochem. Biophys.* **199**, 473–478
- Tenenhouse, H. S., Yip, A. & Jones, G. (1988) *J. Clin. Invest.* **81**, 461–465
- Yamada, S., Nakayama, K., Takayama, H., Shinki, T., Takasaki, Y. & Suda, T. (1984) *J. Biol. Chem.* **259**, 884–889