1,25-Dihydroxyvitamin D_3 Production by Human Keratinocytes

Kinetics and Regulation

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Abstract

Human foreskin keratinocytes in vitro metabolize 25-hydroxyvitamin D_3 to a number of metabolites, including 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). This metabolite remains mostly within the cell and does not accumulate in the medium under the conditions of these experiments. With time, $1,25(OH)_2D_3$ is catabolized, and more polar metabolites appear in both the cells and the medium. The production of $1,25(OH)_2D_3$ has an apparent Michaelis constant (K_m) for 25-hydroxyvitamin D_3 of 5.4×10^{-8} M. The levels of $1,25(OH)_2D_3$ within the cell are increased both by increased production and decreased catabolism when parathyroid hormone(1-34) and isobutylmethylxanthine are added. Exogenously added $1,25(OH)_2D_3$ at concentrations as low as 10^{-12} M reduces endogenous $1,25(OH)_2D_3$ production, increases $1,25(OH)_2D_3$ catabolism, and increases 24,25-dihydroxyvitamin D_3 production by an actinomycin D–sensitive process. These data indicate that the regulation of $1,25(OH)_2D_3$ production by keratinocytes is similar to, but not identical to the regulation of $1,25(OH)_2D_3$ by the kidney.

Introduction

A number of recent studies have suggested that the kidney may not be unique in metabolizing 25-hydroxyvitamin D_3 ($25OHD_3$)[1] to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). The placenta (1–3), bone cells (4, 5), and possibly embryonic intestinal cells (6) produce a metabolite from $25OHD_3$ with the chromatographic properties of $1,25(OH)_2D_3$. Likewise, sarcoid tissue or cells (7, 8) and melanoma cells (9) produce a metabolite thought to be $1,25(OH)_2D_3$. Whether such cells and tissues produce $1,25(OH)_2D_3$ in vivo is unclear. Acutely nephrectomized rats fail to convert $25OHD_3$ to detectable amounts of $1,25(OH)_2D_3$ (10, 11); anephric patients in one study had undetectable $1,25(OH)_2D_3$ levels (12). Detectable levels of this metabolite were recently reported in an anephric patient with sarcoidosis (13), however, and in other anephric patients and anephric pigs given high doses of vitamin D (14, 15). Therefore, anephric humans and pigs may be able to produce $1,25(OH)_2D_3$ in tissues other than the kidney, especially when circulating $25OHD_3$ and parathyroid hormone (PTH) concentrations are high.

The skin is a likely alternate source for $1,25(OH)_2D_3$ because previous investigations have shown that in addition to synthesizing vitamin D_3 (16, 17), the skin can synthesize $24,25(OH)_2D_3$ (18). Furthermore, the skin is well established as a target tissue for $1,25(OH)_2D_3$ because it contains receptors for $1,25(OH)_2D_3$ (19) as well as $1,25(OH)_2D_3$-regulated functions such as vitamin D_3 production and keratin synthesis (18, 20–23). Production of $1,25(OH)_2D_3$ by the skin, therefore, could serve not only a systemic need in times of reduced production of $1,25(OH)_2D_3$ by the kidney, but also a local need in regulating the unique epidermal functions of vitamin D production and keratin synthesis. In recent studies we have observed that human foreskin keratinocytes (but not fibroblasts) produce $1,25(OH)_2D_3$ (24). Identification was based on its elution pattern in several different high performance liquid chromatography (HPLC) systems, its potency in displacing $[^3H]1,25(OH)_2D_3$ from the intestinal cytosol receptor for $1,25(OH)_2D_3$, and its mass spectrum. We present evidence here indicating that the production of $1,25(OH)_2D_3$ by keratinocytes is regulated by substrate ($25OHD_3$) availability, PTH, and $1,25(OH)_2D_3$ in a fashion somewhat similar to the regulation of $1,25(OH)_2D_3$ production by the kidney but with several important differences. These data support the hypothesis that the skin may be an important source of this metabolite, not only for use by the epidermis, but also by the rest of the body when renal production of $1,25(OH)_2D_3$ is impaired.

Methods

Cells. Human newborn foreskins were obtained at circumcision. Keratinocytes were isolated by treatment with trypsin and collagenase and plated onto a monolayer of mitomycin C-treated feeder 3T3 cells in Dulbecco's modified Eagle's medium containing 20% fetal calf serum, penicillin, streptomycin, and amphotericin B, according to the method of Rheinwald and Green (25). Immediately prior to assay, feeder cells were removed from the cultures with 0.1% EDTA. Second or third passage cells were studied after they achieved confluence. All experiments were done on keratinocyte cultures within 1 or 2 d of reaching confluence. At this stage, a few 3T3 cells (<5%) remained attached to the dish under the overlying, multilayered islands of keratinocytes. The relative numbers of 3T3 cells and keratinocytes were ascertained by fluorescence microscopy of cultures after treatment with 1 mg/μl of acridine orange (pH 7.0). Whereas acridine orange labeled the cytosol and nuclei of 3T3 cells orange to red, keratinocytes were easily distinguished by their green-staining cytosol. Moreover, ultrastructural studies on these cultures revealed that virtually all of the cells in confluent cultures were keratinocytes with desmosomes and bundles of intermediate filaments.

After achieving confluence, the protein and DNA content of the culture dishes remained stable for at least 9 d. The mean intraassay coefficients of variation for protein and DNA content from three experiments each with six replicate dishes were 9.9 and 7.3%, respectively.

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1. *Abbreviations used in this paper:* HPLC, high performance liquid chromatography; IBMX, isobutylmethylxanthine; PTH, parathyroid hormone; $25OHD_3$, 25-hydroxyvitamin D_3; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; $24,25(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; $1,24,25(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3.

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The interassay coefficients of variation for protein and DNA content for these three experiments were 4.7 and 3.6%, respectively.

25OHD$_3$ metabolism. Serum-free medium replaced the original medium 40 h prior to the assay of 25OHD$_3$ metabolism. In general, 0.1 μCi 25-hydroxy[26,27-methyl-^{3}H]vitamin D$_3$ (148–153 Ci/mmol, Amersham Corp., Arlington Heights, IL) was added to the culture dish (3 cm diam, 1 ml medium) in 10 μl ethanol. Following the desired period of incubation at 37°C under 1 atmosphere (atm) 5% CO_2 in air, the reaction was stopped with 1 ml methanol. For most experiments, cells and medium were extracted together by the method of Bligh and Dyer (26). The aqueous extract was counted for radioactivity without further processing. The chloroform extract was chromatographed using a Waters HPLC system (Waters Associates, Milford, MA) equipped with a Radiomatics Flo One HP radioactivity detector, a Dupont Zorbax Sil column (4.6 mm × 25 cm) (E. I. Dupont, Wilmington, DE), and a Waters Systems controller to deliver a nonlinear (program 9) gradient from 97:3 to 90:10 hexane/isopropanol at 2 ml/min. The radioactivity monitor determined radioactivity at 6-s intervals, plotted the peaks, summed the radioactivity within each peak, and expressed each sum as a fraction of the total radioactivity in each sample. The results were converted to femtomoles of metabolite per well, based on the specific activity and the amount of [^{3}H]25OHD$_3$ originally added to the incubate. This calculation assumes that each metabolite formed has the same specific activity as the substrate. This assumption would not be valid if tritium loss occurred (as would happen for the production of 25,26-dihydroxyvitamin D$_3$ using the 26,27-labeled 25OHD$_3$ substrate) during the production of the metabolite. Accordingly, for metabolites whose structure is not known, the results must be considered relative and not absolute. Prior to chromatographic analysis of each group of samples, the column was calibrated with the standards 25OHD$_3$, 24(R),25(OH)$_2$D$_3$, 1,25(OH)$_2$D$_3$, and for some experiments with 25(R),26(OH)$_2$D$_3$, 1,24(R),25(OH)$_2$D$_3$, and 1,25(R),26(OH)$_3$D$_3$. The details of each experiment are in the figure legends. In these experiments, recovery of radioactivity was 80±5% in the combined aqueous extract and chromatogram of the chloroform extract.

Identification of metabolites. The presumptive 1,25(OH)$_2$D peak was identified by co-chromatography with chemically synthesized 1,25(OH)$_2$D$_3$ in four different HPLC systems (92 mm × 25 cm μPorasil column eluted with 9:1 hexane/isopropanol; 46 mm × 25 cm Zorbax Sil column eluted with 97:3 to 90:10 hexane/isopropanol gradient or with 96:4 dichloromethane/isopropanol; and 46 mm × 25 cm C18 μBondapak column eluted with 75:25 methanol/water), by its equipotency to chemically synthesized 1,25(OH)$_2$D$_3$ in displacing [^{3}H]1,25(OH)$_2$D from the chick intestinal cytosol receptor, and by mass spectroscopy. These results have been reported elsewhere (24). The presumptive 24,25(OH)$_2$D and 1,24,25(OH)$_2$D peaks have been identified by co-chromatography with chemically synthesized 24,25(OH)$_2$D$_3$ and 1,24(R),25(OH)$_3$D$_3$ on three HPLC systems (92 mm × 25 cm μPorasil column eluted with 9:1 hexane/isopropanol; 46 mm × 25 cm Zorbax Sil column eluted with 97:3 to 90:10 hexane/isopropanol gradient; and C18 μBondapak column eluted with 75:25 methanol/water) and complete (>95%) loss of radioactivity (the metabolites are labeled in the 26,27 position) following periodate cleavage (27).

Other assays. Cells assayed for protein and DNA content were washed with ice-cold phosphate-buffered saline followed by a hypertonic phosphate saline solution (0.05 M NaH_2PO_4, 2.0 M NaCl) containing 2 mM EDTA. The cells were scraped from the plate into the hypertonic saline solution and homogenized with three 15-s bursts of a Fisher sonic dismembrator (model 300, Fisher Scientific, Pittsburgh) at 30% maximal output. The homogenate was stored at −20°C until assay for protein and DNA. The protein determinations were made using the Pierce BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). The DNA determinations were made by the fluorescence assay described by Labarca and Paigen (28).

Statistical analyses. All statistical analyses, including univariable and multivariable analyses of variance, were performed on an IBM-XT computer using the Systat program (Systat Inc., Evanston, IL).

Chemicals. The 25OHD$_3$ was a gift from J. C. Babcock, Upjohn Pharmaceuticals, Kalamazoo, MI; all other vitamin D metabolites were a gift from M. R. Uskokovic, Hoffmann-LaRoche, Nutley, NJ; bovine PTH(1-34) was obtained from Peninsula Laboratories, Belmont, CA. The [^{3}H]1,25(OH)$_2$D$_3$ was biosynthesized from 25OH[26,27-methyl^{3}H]D$_3$ (153 Ci/mmol, Amersham), using the method of Bikle et al. (29). All other chemicals were reagent grade and were obtained from commercial suppliers.

Results

The addition of [^{3}H]25OHD$_3$ to human keratinocytes resulted in rapid uptake (or binding) of radioactivity by the cells that was maximal by 1 h of incubation (Fig. 1). After 1 h, the radioactivity

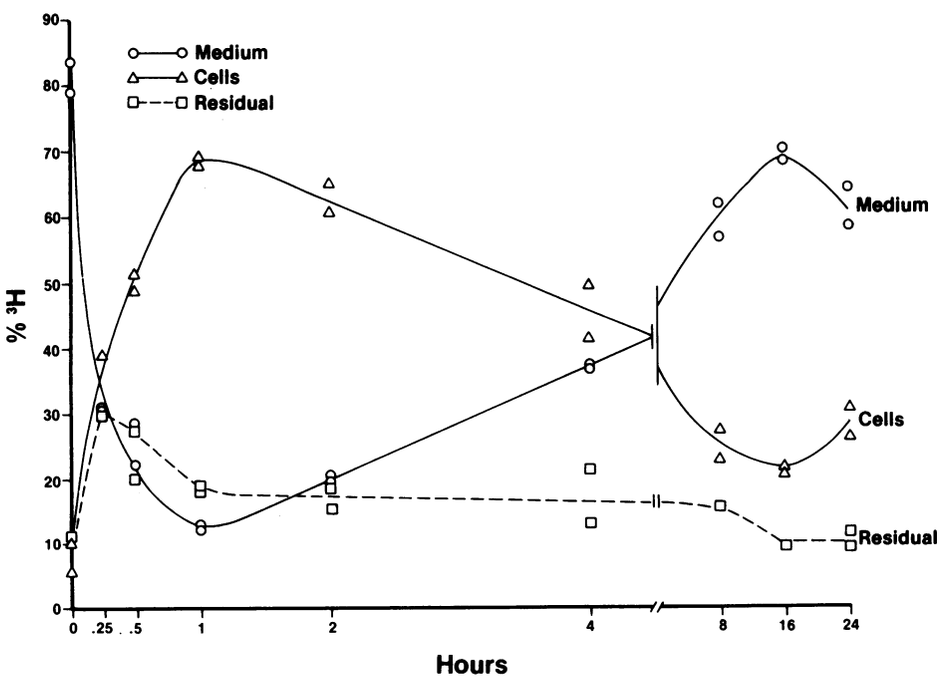

Figure 1. The distribution of radioactivity (^{3}H) between cells and medium as a function of time after the administration of [^{3}H]25OHD$_3$ to the cultured keratinocytes. In this experiment, 18 culture dishes received equal aliquots of [^{3}H]25OHD$_3$. The medium was removed from the duplicate dishes at each of the indicated times after the addition of [^{3}H]25OHD$_3$ and counted for radioactivity. The cells were gently washed once with 1 ml fresh medium (serum free); little radioactivity was present in the wash, so it was discarded. The cells were then scraped into an additional 1 ml of fresh medium, removed from the plate, and counted for radioactivity. The residual radioactivity is that which was extracted by methanol rinses of the dishes after the medium and cells were removed.

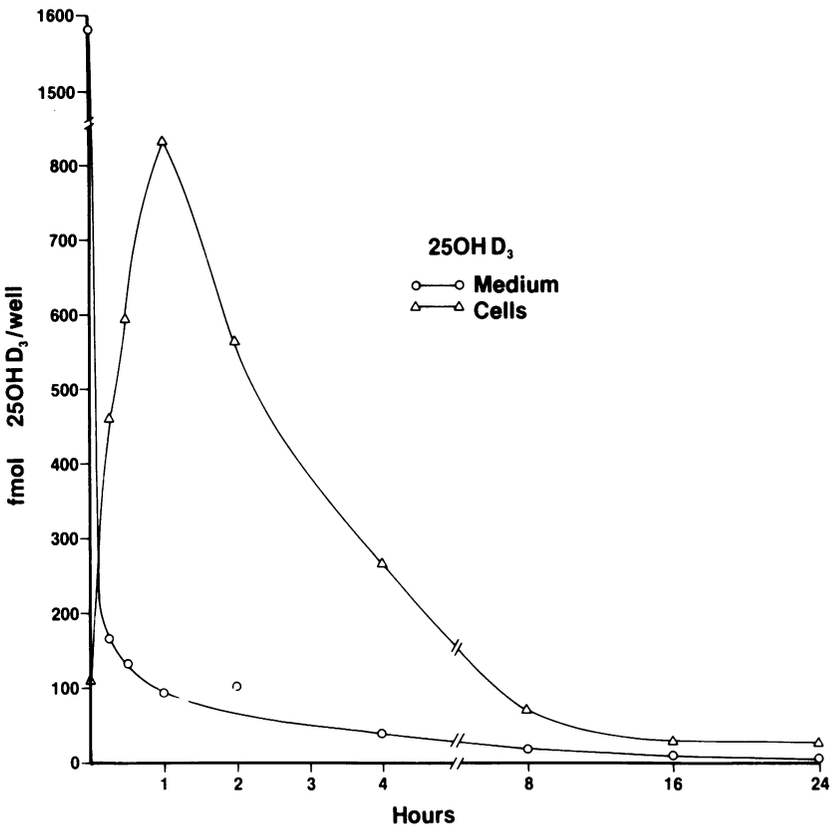

Figure 2. The recovery of $25OHD_3$ from cells and medium as a function of time following the addition of 1,600 fmol [^{3}H]$25OHD_3$ to the culture medium (as in Fig. 1). The medium was removed, and the cells were rinsed once and scraped off into fresh medium. Both cells and medium were extracted separately and analyzed for $25OHD_3$ by HPLC. The mean value of determinations from duplicate wells is shown.

disappeared from the cells and reappeared in the medium. As seen in Fig. 2, most of the radioactivity was in the form of $25OHD_3$ for the first hour. However, very little $25OHD_3$ remained after 24 h. The principal metabolites formed from $25OHD_3$ after 4 h incubation are depicted in the chromatogram in Fig. 3. In this figure, peaks with the elution positions of the

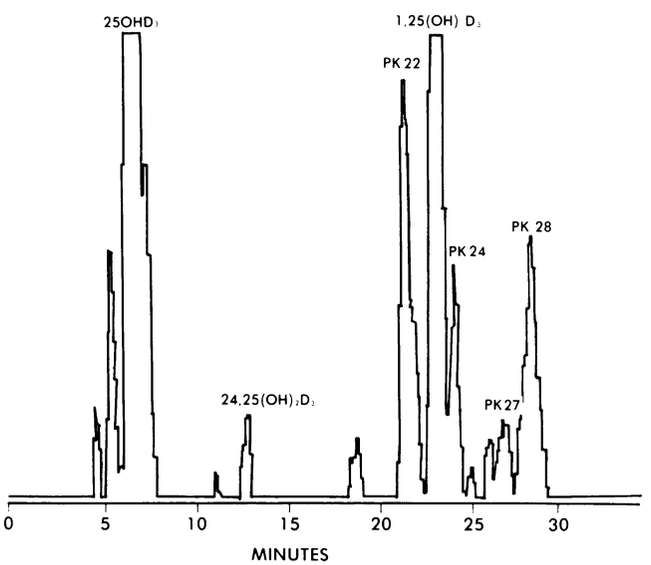

Figure 3. A representative chromatogram of the lipid extract of a keratinocyte culture (cells plus medium) incubated with [^{3}H]$25OHD_3$. This sample was incubated with 1.5×10^{-8} M $25OHD_3$ for 4 h. The peaks eluting in the positions of the standards $25OHD_3$, $24,25(OH)_2D_3$, and $1,25(OH)_2D_3$ are so labeled.

$25OHD_3$, $24,25(OH)_2D_3$, and $1,25(OH)_2D_3$ standards are so labeled. Two minor metabolites eluted earlier than the $25OHD_3$, and another metabolite (minor in this chromatogram) eluted in the position of $24,25(OH)_2D_3$ at 13 min. Other metabolites eluted at approximately 22, 23, 24, 27, and 28 min in this chromatographic system. Only the metabolite eluting at 23 min has been completely identified, and it is $1,25(OH)_2D_3$. The presumptive $24,25(OH)_2D_3$ peak is periodate sensitive, but its structural identity by mass spectroscopy has not been confirmed. The standards $25(R),26(OH)_2D_3$, $1,24(R),25(OH)_2D_3$ and $1,25(R),26(OH)_2D_3$ elute at 21, 31, and 35 min, respectively. Peaks eluting at these times were minor or nonexistent in this experiment, although in other experiments a substantial peak eluting in the position of $1,24,25(OH)_2D_3$ was observed (see description below). This peak also was periodate sensitive. The unidentified metabolites are referred to as peaks 22, 24, 27, and 28 according to their elution time in minutes in this chromatographic system.

The amount of these metabolites recovered from the cells and the medium depended on the duration of the incubation as shown in Fig. 4. A metabolite in peak 22 rapidly appeared in the medium, reaching a peak at 15 min, followed by an exponential decay over the next 24 h. A second metabolite may have appeared in peak 22, increasing in the cell more slowly and reaching a maximum level at 4 h before disappearing by 24 h. The metabolite $1,25(OH)_2D_3$ was found in greatest concentration 1 h after the addition of $25OHD_3$. Very little $1,25(OH)_2D_3$ was released into the medium. The amount of peak 24 in the cells and in the medium increased in parallel (although the cells contained more at all times), reaching a maximum concentration 8 h after the addition of $25OHD_3$. Peak 27 also increased in parallel in both cells and medium, reaching a maximum con-

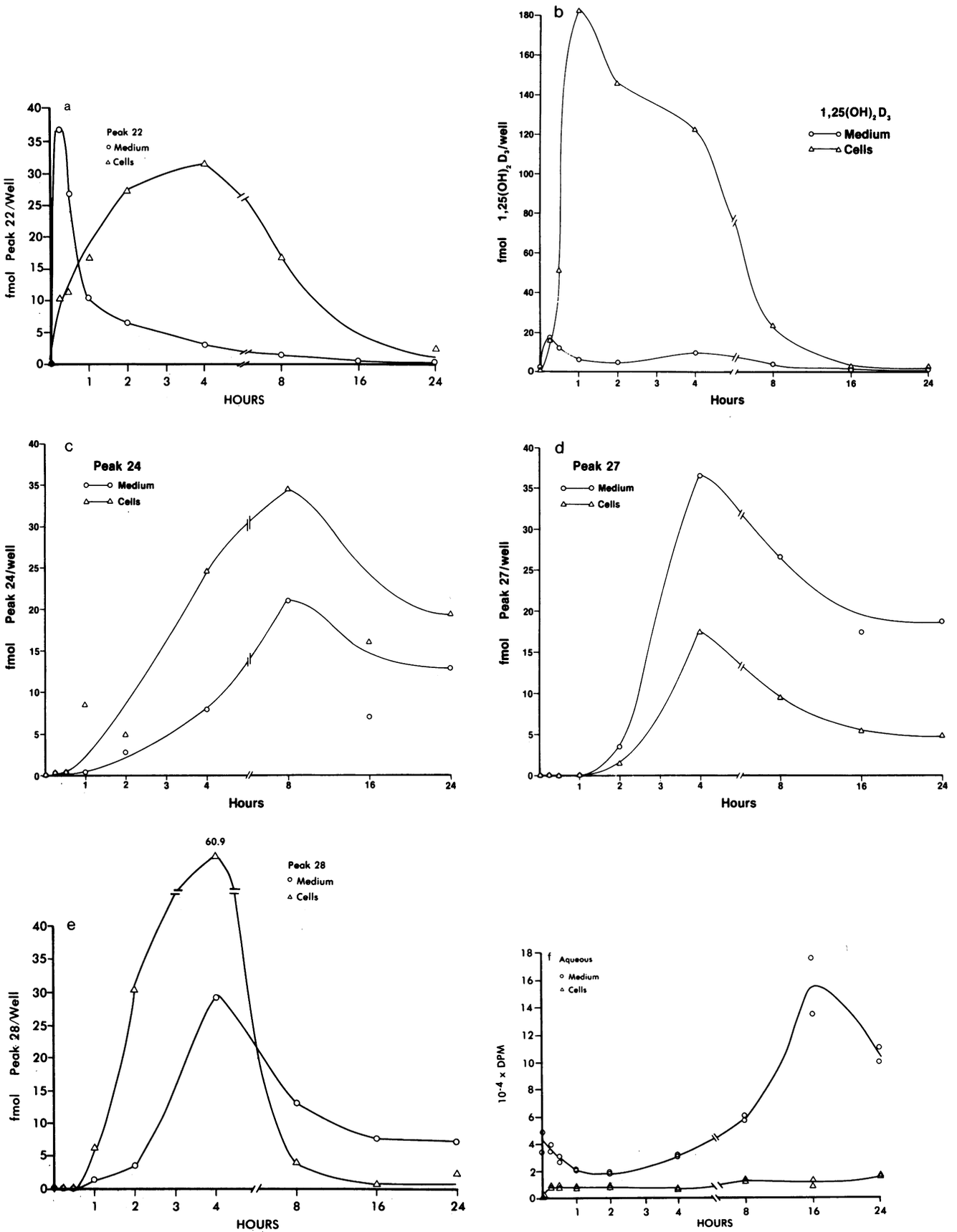

Figure 4. The effect of time of incubation on the recovery of the major metabolites of $25OHD_3$ in cells and medium following the addition of 1,600 fmol [^{3}H]$25OHD_3$ to the culture medium. This experiment illustrates the production and secretion into the medium of the major metabolites of $25OHD_3$ by the keratinocytes. Each point is the mean value of duplicate wells.

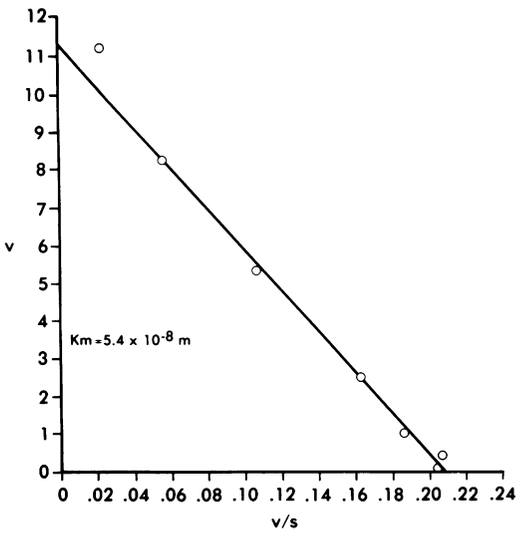

Figure 5. Eadie-Hofstee analysis of $1,25(OH)_2D_3$ production by cultured keratinocytes incubated for 1 h at various $25OHD_3$ concentrations. The velocity (V) units are pmol $1,25(OH)_2D_3$ per h/well; the substrate (S) units are 10^{-9} M. Each point is the mean value of duplicate wells.

centration 4 h after the addition of $25OHD_3$. Unlike peak 24, peak 27 was found in greater amounts in the medium than in the cells. Peak 28 appeared more quickly in the cells than in the medium, although maximum levels were reached in both by 4 h; after 4 h peak 28 disappeared more quickly from cells than from medium. Little $24,25(OH)_2D_3$ or $1,24,25(OH)_3D_3$ was formed in this experiment (data not shown). Little radioactivity was found in the aqueous extract of the cells at any time. Instead, the water-soluble metabolites appeared in the medium where they increased in amount, at least through 16 h of incubation.

The data in Fig. 4 indicate that $1,25(OH)_2D_3$ was the major metabolite of $25OHD_3$ produced by these keratinocytes during the first 4 h of incubation. Maximal levels of $1,25(OH)_2D_3$ (at 1 h) were 190 fmol/well (12% of the original substrate in this experiment), compared to 35 fmol/well of peak 22 (at 4 h), 57 fmol/well of peak 24 (at 8 h), 55 fmol/well of peak 27 (at 4 h), and 90 fmol/well of peak 28 (at 4 h).

The substrate dependence of $1,25(OH)_2D_3$ production was determined using a 1-h incubation and a total $25OHD_3$ concentration from 0.65×10^{-9} M (tracer only) to 500×10^{-9} M. The results analyzed by an Eadie-Hofstee plot (Fig. 5) indicate an apparent K_m for $25OHD_3$ of 5.4×10^{-8} M. This value is only approximate because, as shown in Fig. 2, the actual substrate ($25OHD_3$) concentration available to the intracellular 1α-hydroxylase increased during the 1-h incubation and thus is less than the total substrate concentration upon which these calculations were made. Furthermore, a portion of the $1,25(OH)_2D_3$ produced may have been catabolized during the 1-h incubation so that the amount recovered may have been less than the amount formed (see below). The limited amount of other metabolites formed during the 1 h incubation indicates that catabolism of the $1,25(OH)_2D_3$ was not substantial, however.

Regulation of $25OHD_3$ metabolism was explored. Increasing the calcium concentration in the medium from 0 (calcium free) to 4 mM during incubation had little effect on $25OHD_3$ metabolism (not shown). Likewise, 8-Br-cAMP (0 to 10^{-6} M) or dibutyryl cAMP (0 to 10^{-4} M) had little effect (not shown). However, in the experiments described below (Fig. 6), 1 mM isobutylmethylxanthine (IBMX) increased $1,25(OH)_2D_3$ levels from 94 to 139 fmol/well when added concurrently with [^{3}H]$25OHD_3$, and from 92 to 203 fmol/well when added 4 h prior to a 4-h incubation with [^{3}H]$25OHD_3$.

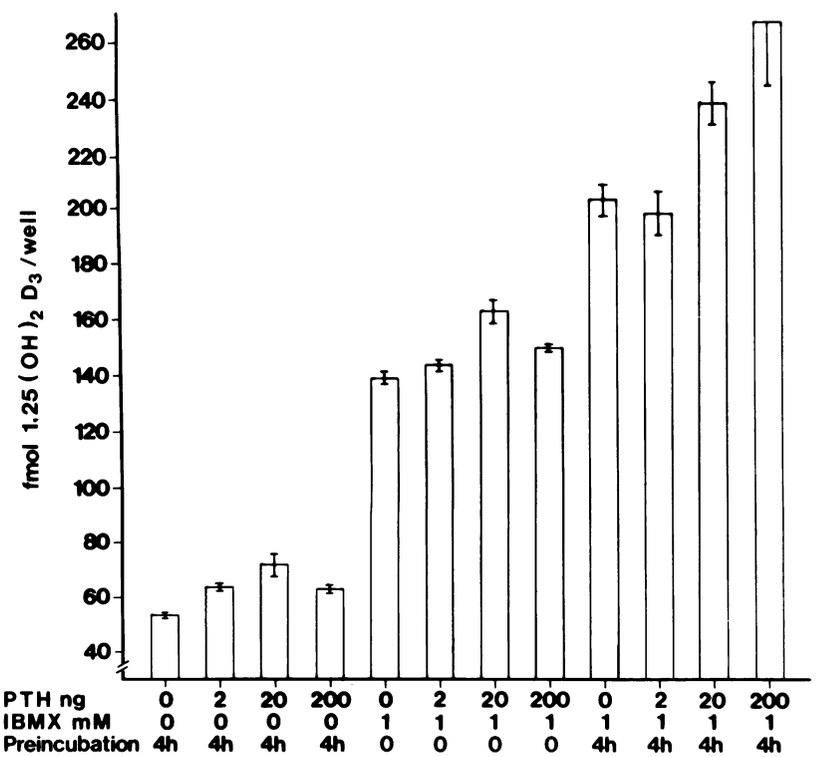

Figure 6. The effect of IBMX and PTH on $1,25(OH)_2D_3$ levels. Three experiments are shown. In the left panel the cells were incubated for 4 h with the indicated concentrations of PTH in the absence of IBMX prior to the addition of 625 fmol [^{3}H]$25OHD_3$ for an additional 4-h incubation. In the middle panel the cells were placed into medium containing 1 mM IBMX and the indicated concentration of PTH at the beginning of the 4-h incubation with 625 fmol of [^{3}H]$25OHD_3$. In the right panel the cells were preincubated with IBMX and the indicated PTH concentration for 4 h before the 4-h incubation with 625 fmol [^{3}H]$25OHD_3$. In all cases, duplicate dishes were run and the data are expressed as mean±range of duplicate determinations. In each of the three experiments, analysis by ANOVA showed that PTH increased $1,25(OH)_2D$ levels significantly ($P = 0.017$, $P < 0.001$, and $P = 0.017$ for the three experiments, respectively). For the combined data, IBMX had a significant ($P < 0.001$) effect on $1,25(OH)_2D$ levels as did preincubation with PTH and IBMX ($P < 0.001$).

PTH increased $1,25(OH)_2D_3$ levels after a 4-h incubation with [^{3}H]25OHD$_3$ (Fig. 6). In the absence of IBMX, increasing the PTH concentrations from 0 to 20 ng/ml raised $1,25(OH)_2D_3$ levels 33% from 54 to 72 fmol/well. Higher concentrations of PTH (200 ng/ml) were less effective. When PTH was added with IBMX at the start of the 4-h incubation with [^{3}H]25OHD$_3$, it enhanced the stimulatory effect of IBMX; again at the optimal concentration of 20 ng/ml, 163 fmol $1,25(OH)_2D_3$/well were recovered in comparison to 139 fmol/well in the presence of IBMX alone. When the cells were incubated for 4 h with IBMX and PTH before [^{3}H]25OHD$_3$ was added, the 200-ng/ml PTH concentration was optimal in increasing $1,25(OH)_2D_3$ levels, raising them from 203 fmol/well in the absence of PTH to 266 fmol/well in the presence of PTH. In this experiment the recovery of 266 fmol of $1,25(OH)_2D_3$ represented 43% of the initial substrate added (625 fmol of [^{3}H]25OHD$_3$).

The effects of IBMX and PTH on the levels of $1,25(OH)_2D_3$ were associated with marked changes in the levels of other metabolites. In particular, the amount of peak 28 showed a reciprocal relationship to the amount of $1,25(OH)_2D_3$ recovered during these 4-h incubations with [^{3}H]25OHD$_3$. The combination of PTH and IBMX markedly inhibited the appearance of peak 28. These effects on $1,25(OH)_2D_3$ levels thus are consistent either with a stimulation by PTH and IBMX of $1,25(OH)_2D_3$ production or with an inhibition by PTH and IBMX of $1,25(OH)_2D_3$ catabolism to other metabolites (such as peak 28).

To determine how PTH and IBMX raise $1,25(OH)_2D_3$ levels in keratinocytes we performed two types of experiments. First we determined the effects of PTH and IBMX on 25OHD$_3$ metabolism at three different times of incubation: 1, 4, and 8 h. The results for all major metabolites of 25OHD$_3$ formed in this experiment are shown in Table I. In this experiment metabolites eluting in the position of $24,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$ were observed. At 1 h of incubation, $1,25(OH)_2D_3$ was the major metabolite formed, and its production was stimulated from 70 to 94 fmol/well by 20 ng/ml PTH and from 70 to 147 fmol/well by IBMX. No detectable peak 28 was formed by 1 h. Levels of $24,25(OH)_2D_3$ were little affected by PTH but were abolished by IBMX, whereas $1,24,25(OH)_2D_3$ levels were increased from 2.3 to 4.7 fmol/well by PTH and to 11 fmol/well by IBMX. After a 4-h incubation with [^{3}H]25OHD$_3$, peak 28 and $1,24,25(OH)_3D_3$ were the major metabolites recovered (113 and 89 fmol/well, respectively) although substantial amounts of $1,25(OH)_2D_3$ (54 fmol/well) were still present. PTH increased the levels of both $1,25(OH)_2D_3$ (to 79 fmol/well) and $1,24,25(OH)_3D_3$ (to 110 fmol/well) with little effect on the other metabolites. IBMX increased $1,25(OH)_2D_3$ levels to 231 fmol/well (a 427% increase over controls), reduced $24,25(OH)_2D_3$ levels from 23 to 2 fmol/well, reduced peak 28 levels from 113 to 14 fmol/well, and reduced $1,24,25(OH)_3D_3$ levels from 89 to 46 fmol/well. After an 8-h incubation, most of the radioactive label was in the form of water-soluble metabolites (263 fmol equivalents/well). IBMX markedly inhibited the production of the water-soluble metabolites (reducing them from 263 to 113 fmol

Table I. Time Course of PTH and IBMX Effects on 25OHD Metabolism

		fmol Recovered						
PTH	IBMX	$24,25(OH)_2D_3$	Peak 22	$1,25(OH)_2D_3$	Peak 24	Peak 28	$1,24,25(OH)_3D_3$	Aqueous
ng/ml	*nM*							
1 h Incubation								
0	0	4.7±0.9	3.1±0.1	70±6	1.6±0.3	—	2.3±0.1	7.2±0.3
20	0	3.1±0.6	1.9±0.6	94±9	1.3±0.1	—	4.7±0.9	11±0.9
0	1	—	5.0±1.9	147±9	1.6±0.9	—	11±4	13±0.3
20	1	—	1.9±1.9	144±4	2.5±1.2	—	12±4	18±2
4 h Incubation								
0	0	23±0.6	28±3	54±0.6	36±3	113±6	89±1	49±1
20	0	23±6	31±6	79±8	34±6	102±0.3	110±2	53±3
0	1	2±1	23±4	231±5	10±0.9	13±5	46±12	36±2
20	1	1.3±0.6	33±3	274±9	12±2	12±1	50±3	38±3
8 h Incubation								
0	0	17±1	11±0.1	28±0.6	29±2	33±2	14±0.9	263±8
20	0	21±2	23±6	34±5	22±4	43±3	11±3	275±10
0	1	32±8	61±6	28±0.6	25±9	91±11	21±1	113±13
20	1	25±6	52±3	48±6	33±0.3	106±34	62±18	102±20

In this experiment the cells were preincubated with the indicated concentrations of PTH and IBMX for 4 h prior to the addition of 625 fmol [^{3}H]25OHD$_3$. The incubation time refers to the duration of the incubation following the addition of [^{3}H]25OHD$_3$. Each incubation condition was performed in duplicate and the results are expressed as fmol/well ± range of duplicates. A line (—) indicates no detectable metabolite. Multivariate ANOVA analysis showed the following results: $24,25(OH)_2D$ levels were significantly increased with time of incubation ($P < 0.001$) and decreased with IBMX ($P = 0.019$); peak 22 levels were significantly increased with time of incubation ($P < 0.001$) and IBMX ($P < 0.001$); $1,25(OH)_2D$ levels were significantly decreased with time of incubation ($P < 0.001$) but increased with PTH ($P < 0.001$) and IBMX ($P < 0.001$); levels of peaks 24 and 28 were significantly increased with time of incubation ($P < 0.001$); $1,24,25(OH)_2D$ levels were significantly increased with time of incubation ($P < 0.001$) and with PTH ($P = 0.042$); aqueous radioactivity was significantly increased with time of incubation ($P < 0.001$) and decreased with IBMX ($P < 0.001$).

equivalents/well), which resulted in increased levels of the chloroform extractable metabolites, including peak 28. PTH in the presence of IBMX selectively increased the recovery of $1,25(OH)_2D_3$ (from 28 to 48 fmol/well) and $1,24,25(OH)_3D_3$ (from 21 to 62 fmol/well) in the cells incubated with [^{3}H]25OHD$_3$ for 8 h.

The second type of experiment directly assessed the effects of IBMX and PTH on the metabolism of $1,25(OH)_2D_3$. We added radioactively labeled $1,25(OH)_2D_3$ to keratinocytes following a 4-h incubation with 20 ng/ml PTH or 1 mM IBMX for an additional 4-h incubation. The results of this experiment are shown in Table II. In this experiment, only 29% of the added [^{3}H]$1,25(OH)_2D_3$ was recovered as $1,25(OH)_2D_3$ in the absence of PTH and IBMX. 30% appeared in peak 28 and 22% appeared as $1,24,25(OH)_3D_3$. PTH had little effect on the metabolism of $1,25(OH)_2D_3$ in the absence of IBMX. In contrast, the inclusion of IBMX in the medium resulted in substantially less metabolism of $1,25(OH)_2D_3$. 47% of the added [^{3}H]$1,25(OH)_2D_3$ was recovered as $1,25(OH)_2D_3$; the percent recovered in peak 28 fell from 31 to 10.5%, whereas the amount recovered as $1,24,25(OH)_3D_3$ was not changed. The addition of PTH in the presence of IBMX further increased the recovery of $1,25(OH)_2D_3$ to 59% and reduced the conversion of $1,25(OH)_2D_3$ to peak 28 (6.6%) and $1,24,25(OH)_3D_3$ (14%). These results suggest that IBMX raises $1,25(OH)_2D_3$ levels in keratinocytes by retarding the catabolism of $1,25(OH)_2D_3$. PTH, on the other hand, appears to increase $1,25(OH)_2D_3$ (and $1,24,25(OH)_3D_3$) levels primarily by stimulating the 1α-hydroxylase, but also has a synergistic effect with IBMX to retard $1,25(OH)_2D_3$ catabolism.

We then examined the ability of exogenous $1,25(OH)_2D_3$ to regulate its own production. When $1,25(OH)_2D_3$ was added to keratinocyte cultures 16 h prior to the addition of 676 fmol [^{3}H]25OHD$_3$, the recovery of [^{3}H]$1,25(OH)_2D_3$ was markedly reduced (Fig. 7). At the lowest dose tested (10^{-12} M), the recovery of [^{3}H]$1,25(OH)_2D_3$ (75 fmol/well) was 83% of that found in the absence of exogenous $1,25(OH)_2D_3$ (90 fmol/well), and at 10^{-11} M, the recovery of [^{3}H]$1,25(OH)_2D_3$ was reduced by 50%. In contrast, the recovery of [^{3}H]$24,25(OH)_2D_3$ was increased from 10 to 28 fmol/well by the presence of 10^{-11} M $1,25(OH)_2D_3$. As the concentration of exogenous $1,25(OH)_2D_3$ increased, the recovery of [^{3}H]$1,25(OH)_2D_3$ fell to essentially zero, while the recovery of [^{3}H]$24,25(OH)_2D_3$ continued to increase, reaching 48 fmol/well at 10^{-8} M $1,25(OH)_2D_3$. The recovery of [^{3}H]$1,24,25(OH)_3D_3$ was increased (from 11 to 27 fmol/well) by increasing $1,25(OH)_2D_3$ from 0 to 10^{-10} M, but at higher concentrations of exogenous $1,25(OH)_2D_3$, the recovery of this metabolite fell in parallel with the fall in recovery of [^{3}H]$1,25(OH)_2D_3$. The amount of radioactivity recovered from the aqueous extract (38 fmol equivalents/well in controls) was increased by concentrations of exogenous $1,25(OH)_2D_3$ greater than 10^{-12} M, reaching a level of 98 fmol equivalents/well at 10^{-8} M $1,25(OH)_2D_3$. Similar results were obtained when a 4-h incubation with [^{3}H]25OHD$_3$ was used except that 10^{-12} M exogenous $1,25(OH)_2D_3$ was sufficient to reduce recoverable [^{3}H]$1,25(OH)_2D_3$ to 50% of control (data not shown). None of these changes induced by $1,25(OH)_2D_3$ was found when actinomycin D (2 μg/ml) was added to the medium at the time $1,25(OH)_2D_3$ was added (data not shown). Actinomycin D markedly reduced [^{3}H]$1,25(OH)_2D_3$ recovery (to 11 fmol/well) in all cultures regardless of the presence of exogenous $1,25(OH)_2D_3$. In a parallel experiment, incubation of keratinocytes with these same concentrations of $1,25(OH)_2D$ did not significantly decrease the protein or DNA content of the cultures or alter the extent to which the cells excluded trypan blue.

Because of the possibility that exogenous $1,25(OH)_2D_3$ might affect its own catabolism as well as production, we incubated [^{3}H]$1,25(OH)_2D_3$ for 1 h with control cells or with cells previously exposed to 10^{-8} M $1,25(OH)_2D_3$ for 16 h. The results are shown in Table III. Even without preincubation with $1,25(OH)_2D_3$, only 61% of the added [^{3}H]$1,25(OH)_2D_3$ could be recovered as

Table II. Effect of PTH and IBMX on $1,25(OH)_2D_3$ Metabolism

		% Recovery			
PTH	IBMX	$1,25(OH)_2D_3$	Peak 28	$1,24,25(OH)_3D_3$	Aqueous
0	0	28.9±1.5	30.7±1.2	21.8±0.8	8.5±0.5
20 ng/ml	0	28.5±4.0	28.2±1.0	21.2±0.7	7.3±2.9
0	1 mM	46.9±3.3	10.5±0.9	21.0±4.7	4.6±0.3
20 ng/ml	1 mM	58.5±8.0	6.6±0.4	14.4±1.0	4.6±0.3

In this experiment the cells were incubated with the indicated concentration of PTH and/or IBMX for 4 h at which point 0.05 μCi [^{3}H]$1,25(OH)_2D_3$ was added for an additional 4 h incubation. The data are expressed as percent of the label recovered in the indicated peak following HPLC of the chloroform extract or in the aqueous extract. Each incubation was performed in duplicate, and the mean±range of duplicates is shown. Multivariate ANOVA analysis showed that only IBMX (not PTH) significantly increased the levels of $1,25(OH)_2D$ ($P = 0.008$) while decreasing the levels of peak 28 ($P < 0.001$) and aqueous radioactivity ($P = 0.05$).

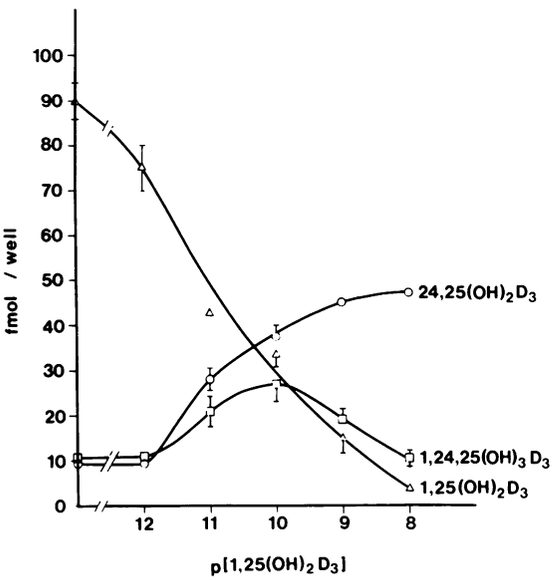

Figure 7. The effect of $1,25(OH)_2D$ on 25OHD metabolism by keratinocytes. Keratinocytes were incubated with nonradioactively labeled $1,25(OH)_2D_3$ at the indicated concentrations (p[$1,25(OH)_2D_3$] is the negative log of [$1,25(OH)_2D_3$]) for 16 h. The medium was removed, the cells were washed once with fresh medium and were then incubated with [^{3}H]25OHD for 1 h. The production of $1,25(OH)_2D_3$ (△), $24,25(OH)_2D_3$ (○), and $1,24,25(OH)_2D_3$ (□) is shown. Each point is the mean value±range of duplicate determinations. ANOVA analysis showed that $1,25(OH)_2D$ significantly decreased $1,25(OH)_2D$ levels ($P < 0.001$), significantly increased $24,25(OH)_2D$ levels ($P < 0.001$), and significantly increased $1,24,25(OH)_2D_3$ levels ($P = 0.029$).

Table III. Metabolism of [^{3}H]1,25(OH)$_2$D$_3$ by Keratinocytes as Affected by Prior Incubation with 10^{-8} M Nonradioactive 1,25(OH)$_2$D$_3$

	% Recovery			
	1,25(OH)$_2$D$_3$	Peak 28	1,24,25(OH)$_2$D$_3$	Aqueous
Control				
1,25(OH)$_2$D$_3$	60.7±2.3	10.4±0.5	12.9±0.7	8.0±0.8
	20.5±4.4	27.8±2.1	19.4±2.5	27.0±1.9

0.05 μCi [^{3}H]1,25(OH)$_2$D$_3$ was incubated for 1 h with keratinocytes preincubated for 16 h with either vehicle (control) or 10^{-8} M nonradioactive 1,25(OH)$_2$D$_3$. The data are shown as the percent recovery of radioactivity in the various peaks following HPLC of the chloroform extract or in the aqueous extract. Each incubation was performed in duplicate and the data are given as mean±range of duplicates. ANOVA analysis showed that 1,25(OH)$_2$D preincubation significantly decreased the recovery of radiolabeled 1,25(OH)$_2$D ($P = 0.015$) and increased the recovery of peak 28 ($P = 0.015$) and aqueous radioactivity ($P = 0.012$).

1,25(OH)$_2$D$_3$ after a 1-h incubation; 10.4% of the radioactive label appeared as peak 28 and 13% as 1,24,25(OH)$_2$D$_3$. Preincubation with nonlabeled 1,25(OH)$_2$D$_3$ markedly enhanced the ability of the cells to metabolize [^{3}H]1,25(OH)$_2$D$_3$ to both peak 28 (28%) and 1,24,25(OH)$_3$D$_3$ (19%); only 20% of the added [^{3}H]1,25(OH)$_2$D$_3$ could be recovered as 1,25(OH)$_2$D$_3$. In both control and 1,25(OH)$_2$D$_3$-treated cells, small amounts (<5%) of the radioactive label appeared in peaks 22, 24, and 27 (not shown).

Discussion

Because the skin is the source of vitamin D, and vitamin D production may be regulated by the biologically active vitamin D metabolites (20), we evaluated whether skin cells (keratinocytes) could produce these active vitamin D metabolites. We observed that the keratinocytes did metabolize 25OHD$_3$ to 1,25(OH)$_2$D$_3$, a metabolite that we identified by chromatography by its affinity for the intestinal cytosol 1,25(OH)$_2$D$_3$ receptor, and by mass spectroscopy (24). The keratinocytes also metabolized 25OHD$_3$ to other metabolites that are not yet fully identified. The experiments reported here represent our efforts to assess the regulation of 1,25(OH)$_2$D$_3$ production and catabolism by these cells.

We observed that the production of 1,25(OH)$_2$D$_3$ by the keratinocytes is brisk and apparently linear with time for the first hour of incubation with 25OHD$_3$. After 1 h, the levels of 1,25(OH)$_2$D$_3$ decline as other metabolites are produced. The direct addition of 1,25(OH)$_2$D$_3$ to these cells indicates that at least some of these metabolites are produced from 1,25(OH)$_2$D$_3$ itself. The time course of 1,25(OH)$_2$D$_3$ production by primary renal cell cultures has not been well described, but one report (30) indicates that 7 h was required for maximal conversion of 25OHD$_3$ to 1,25(OH)$_2$D$_3$. These differences in time course suggest substantial differences in metabolic rates between keratinocytes and renal cells with respect to vitamin D metabolism.

The apparent K_m of the 1α-hydroxylase in keratinocytes for 25OHD$_3$ was found to be 5.4×10^{-8} M. This value is within the range reported in two studies for the 1α-hydroxylase in primary renal cell cultures—1.3×10^{-8} M (30) and 12.5×10^{-8} M (31)—and below that reported for freshly isolated renal cell tubules and mitochondria, 32×10^{-8} M (32). In all these preparations the apparent K_m is only an estimate of the true K_m because of the finite amount of time required to transport the substrate (25OHD$_3$) to the enzyme, and because the product (1,25(OH)$_2$D$_3$) continued to be metabolized. If one assumes that these estimates are reasonable approximations of the true K_m, and if only the free 25OHD$_3$ concentration in blood is available to the 1α-hydroxylase (which we have measured as 0.015% of the total 25OHD$_3$, or ~15 pM in normal individuals, manuscript in preparation), then the 1α-hydroxylase in kidney and skin should be undersaturated and able to produce 1,25(OH)$_2$D$_3$ almost linearly as the available 25OHD$_3$ substrate is increased. However, this does not seem to occur in vivo in normal individuals, suggesting that the 1α-hydroxylase is regulated by mechanisms other than the substrate availability.

PTH, 1,25(OH)$_2$D$_3$, and calcium are well-established regulators of 1,25(OH)$_2$D$_3$ production by the kidney in vivo. The effects of calcium on 1,25(OH)$_2$D$_3$ production by intact renal cells in vitro have been subtle (30–32), so our failure to demonstrate an effect of extracellular calcium on the production of 1,25(OH)$_2$D$_3$ by keratinocytes was not unexpected. Perhaps the extracellular calcium concentration had little influence on the intracellular calcium concentration in these experiments. However, a number of studies have reported that PTH stimulates 1,25(OH)$_2$D$_3$ production by intact renal cells in vitro (30, 31, 33–37). Stimulation of the renal 1α-hydroxylase in these reports required 2–200 ng/ml PTH, the same concentration range that increased the amount of 1,25(OH)$_2$D$_3$ in keratinocytes incubated with 25OHD$_3$. IBMX, a potent phosphodiesterase inhibitor, also increased the amount of 1,25(OH)$_2$D$_3$ recovered from the keratinocytes despite our inability to demonstrate an effect of cAMP. The increase in recoverable 1,25(OH)$_2$D$_3$ in cells incubated with IBMX and PTH was greater if the IBMX and PTH were added 4 h before the 4-h incubation with 25OHD$_3$. This result contrasts with our results in freshly isolated renal cells and slices (34, 35) in which the maximal effect of PTH was acute; however, in other studies using cultured renal cells, the effects of PTH on 1,25(OH)$_2$D$_3$ production also required several hours (30, 31, 36).

Further exploration into the mechanism by which PTH and IBMX raised 1,25(OH)$_2$D$_3$ levels in the keratinocytes revealed that IBMX markedly retarded the further metabolism of 1,25(OH)$_2$D$_3$ especially to an unidentified metabolite (peak 28) that eluted after 1,25(OH)$_2$D$_3$ but before 1,24,25(OH)$_3$D$_3$. The very high levels of 1,25(OH)$_2$D$_3$ reached in the keratinocytes after 4 h of incubation with 25OHD$_3$ in the presence of IBMX thus can be accounted for by the ability of IBMX to block the catabolism of 1,25(OH)$_2$D$_3$. PTH may potentiate this effect of IBMX, but it also appears to directly stimulate 1,25(OH)$_2$D$_3$ production since it raised the 1,25(OH)$_2$D$_3$ levels without reducing the levels of other metabolites (such as peak 28) in the absence of IBMX. The ability of PTH to raise 1,25(OH)$_2$D$_3$ levels is modest compared with the ability of IBMX, but the degree of stimulation is comparable with that achieved using a variety of renal preparations.

1,25(OH)$_2$D$_3$ is a potent inhibitor of its own production by renal cells. However, even in serum-free medium, the concentration of this metabolite required to inhibit its own production

by 50% is at least 10^{-9} M (31, 37, 38). In contrast, the amount of radioactively labeled $1,25(OH)_2D_3$ recovered from the keratinocytes after a 1-h incubation with $[^3H]25OHD_3$ was reduced by 50% at 10^{-11} M exogenous $1,25(OH)_2D_3$, with significant inhibition at 10^{-12} M. In other experiments in which a 4-h incubation with $[^3H]25OHD_3$ was employed, 10^{-12} M $1,25(OH)_2D_3$ was sufficient to reduce recoverable $[^3H]1,25(OH)_2D_3$ to 50% of control values. At least part of this effect appears to be due to further metabolism of the newly produced $1,25(OH)_2D_3$ following pretreatment of the cells with exogenous $1,25(OH)_2D_3$. As for the experiments with PTH and IBMX, peaks 28 and $1,24,25(OH)_2D_3$ are the major metabolites of $1,25(OH)_2D_3$ under these conditions.

These data suggest that $1,25(OH)_2D_3$ production by keratinocytes is more sensitive to inhibition by exogenous $1,25(OH)_2D_3$ than is the renal production of $1,25(OH)_2D_3$. Our (29) recent report that the free concentration of $1,25(OH)_2D_3$ in human sera is $\sim 0.5 \times 10^{-12}$ M suggests that at normal circulating levels of free $1,25(OH)_2D_3$, production of that metabolite by epidermal cells may be more inhibited than is its production by renal tubules. Catabolism of $1,25(OH)_2D_3$ may also be more sensitive to $1,25(OH)_2D_3$ in epidermal cells than in renal tubules, but data from such cells regarding this point have not been reported.

The demonstration that human foreskin keratinocytes produce $1,25(OH)_2D_3$ from $25OHD_3$ in vitro raises the question of whether the skin is a source of $1,25(OH)_2D_3$ in vivo. Our in vitro data suggest that under physiologic circumstances, the skin may contribute little to circulating $1,25(OH)_2D$ levels for two reasons: (*a*) at circulating levels of $1,25(OH)_2D$, production of this metabolite by the skin may be inhibited and its catabolism may be stimulated, and (*b*) secretion of $1,25(OH)_2D$ into the blood from the skin may be limited. Conceivably, most of the $1,25(OH)_2D_3$ formed by the keratinocytes serves a function within the cell in which it is produced. Further investigation is necessary to resolve this important issue.

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References

1. Gray, T. K., G. E. Lester, and R. S. Lorenc. 1979. Evidence for extra-renal 1α-hydroxylation of 25-hydroxyvitamin D_3 in pregnancy. *Science (Wash. DC).* 204:1311–1312.

2. Whitsett, J. A., M. Ho, R. C. Tsang, E. J. Norman, and K. G. Adams. 1981. Synthesis of 1,25-dihydroxyvitamin D_3 by human placenta in vitro. *J. Clin. Endocrinol. Metab.* 53:484–488.

3. Weisman, Y., A. Harell, S. Edelstein, M. David, Z. Spirer, and A. Golander. 1979. 1α,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 in vitro synthesis by human decidua and placenta. *Nature (Lond.).* 281:317–319.

4. Howard, G. A., R. T. Turner, D. J. Sherrard, and D. J. Baylink. 1981. Human bone cells in culture metabolize 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3. *J. Biol. Chem.* 256:7738–7740.

5. Turner, R. T., J. E. Puzas, M. D. Forte, G. E. Lester, T. K. Gray, G. A. Howard, and D. J. Baylink. 1980. In vitro synthesis of 1α,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol by isolated calvarial cells. *Proc. Natl. Acad. Sci. USA.* 77:5720–5724.

6. Puzas, J. E., R. T. Turner, G. A. Howard, and D. J. Baylink. 1983. Cells isolated from embryonic intestine synthesize 1,25-dihydroxyvitamin-D_3 and 24,25-dihydroxyvitamin-D_3 in culture. *Endocrinology.* 112: 378–380.

7. Mason, R. S., T. Frankel, Y-L. Chan, D. Lissner, and S. Posen. 1984. Vitamin D conversion by sarcoid lymph node homogenate. *Ann. Intern. Med.* 100:59–61.

8. Adams, J. S., O. P. Sharma, M. A. Gacad, and F. R. Singer. 1983. Metabolism of 25-hydroxyvitamin D_3 by cultured pulmonary alveolar macrophages in sarcoidosis. *J. Clin. Invest.* 72:1856–1860.

9. Frankel, T. L., R. S. Mason, P. Hersey, E. Murray, and S. Posen. 1983. The synthesis of vitamin D metabolites by human melanoma cells. *J. Clin. Endocrinol. Metab.* 57:627–631.

10. Reeve, L., Y. Tanaka, and H. F. DeLuca. 1983. Studies on the site of 1,25-dihydroxyvitamin D_3 synthesis in vivo. *J. Biol. Chem.* 258: 3615–3617.

11. Shultz, T. D., J. Fox, H. Heath III, and R. Kumar. 1983. Do tissues other than the kidney produce 1,25-dihydroxyvitamin D_3 in vivo? A re-examination. *Proc. Natl. Acad. Sci. USA.* 80:1746–1750.

12. Brumbaugh, P. F., D. H. Haussler, K. M. Bursac, and M. R. Haussler. 1974. Filter assay for 1α,25-dihydroxyvitamin D_3. Utilization of the hormone's target tissue chromatin receptor. *Biochemistry.* 13: 4091–4097.

13. Barbour, G. L., J. W. Coburn, E. Slatopolsky, A. W. Norman, and R. L. Horst. 1981. Hypercalcemia in an anephric patient with sarcoidosis: evidence for extrarenal generation of 1,25-dihydroxyvitamin D_3. *N. Engl. J. Med.* 305:440–443.

14. Lambert, P. W., P. H. Stern, R. C. Avioli, N. C. Brackett, R. T. Turner, A. Greene, I. Y. Fu, and N. H. Bell. 1982. Evidence for extrarenal production of 1α,25-dihydroxyvitamin D in man. *J. Clin. Invest.* 69: 722–725.

15. Littledike, E. T., and R. L. Horst. 1982. Metabolism of vitamin D_3 in nephrectomized pigs given pharmacological amounts of vitamin D_3. *Endocrinology.* 111:2008–2013.

16. Holick, M. F. 1981. The cutaneous photosynthesis of previtamin D_3: a unique photoendocrine system. *J. Invest. Dermatol.* 77:51–58.

17. Nemanic, M. K., J. Whitney, S. Arnaud, S. Herbert, and P. M. Elias. 1983. Vitamin D_3 production by cultured human keratinocytes and fibroblasts. *Biochem. Biophys. Res. Comm.* 115:444–450.

18. Feldman, D., T. Chen, C. Cone, M. Hurst, S. Shani, A. Benderli, and A. Hockberg. 1982. Vitamin D resistent rickets with alopecia: cultured skin fibroblasts exhibit defective cytoplasmic receptors and unresponsiveness to $1,25(OH)_2D_3$. *J. Clin. Endocrinol. Metab.* 55:1020–1022.

19. Feldman, D., T. Chen, M. Hirst, K. Colston, M. Karasek, and C. Cone. 1980. Demonstration of 1,25-dihydroxyvitamin D_3 receptors in human skin biopsies. *J. Clin. Endocrinol. Metab.* 51:1463–1465.

20. Nemanic, M. K., D. D. Bikle, and P. M. Elias. 1983. Hormonal regulation of vitamin D_3 production in human foreskin keratinocytes. *Calcif. Tissue Int.* 35:700.

21. Esvelt, R. P., H. F. DeLuca, J. K. Wichmann, S. Yoshizawa, J. Zurcher, M. San, and W. E. Stumpf. 1980. 1,25-dihydroxyvitamin D_3-stimulated increase of 7,8-didehydroxycholesterol levels in rat skin. *Biochemistry.* 19:6158–6161.

22. Hosomi, J., J. Hosoi, E. Abe, T. Suda, and T. Kuroki. 1983. Regulation of terminal differentiation of cultured mouse epidermal cells by 1α,25-dihydroxyvitamin D_3. *Endocrinology.* 113:1950–1957.

23. Clemens, T. L., J. S. Adams, N. Horiuchi, B. A. Gilchrist, H. Cho, Y. Tsuchiza, N. Matsuo, T. Suda, and M. F. Holick. 1983. Interaction of 1,25-dihydroxyvitamin D_3 with keratinocytes and fibroblasts from skin of normal subjects and a subject with vitamin D-dependent rickets, type II: a model for the study of the mode of action of 1,25-dihydroxyvitamin D_3. *J. Clin. Endocrinol. Metab.* 56:824–830.

24. Bikle, D. D., M. K. Nemanic, J. O. Whitney, and P. W. Elias. 1986. Neonatal human foreskin keratinocytes produce 1,25-dihydroxyvitamin D_3. *Biochemistry.* 25:1545–1548.

25. Rheinwald, J., and H. Green. 1975. Serial cultivation of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell.* 6:331–343.

26. Bligh, E. G., and W. G. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917.

27. Bikle, D. D., and H. Rasmussen. 1974. The metabolism of 25-hydroxycholecalciferol by isolated renal tubules as studied by a new chromatographic technique. *Biochim. Biophys. Acta.* 362:425–438.

28. Labarca, C., and K. Paigen. 1980. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* 102:344–352.

29. Bikle, D. D., E. Gee, B. Halloran, and J. G. Haddad. 1984. Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects and subjects with liver disease. *J. Clin. Invest.* 74:1966–1971.

30. Fukase, M., S. J. Birge, Jr., L. Rifas, L. V. Avioli, and L. R. Chase. 1982. Regulation of 25-hydroxyvitamin D_3 1-hydroxylase in serum-free monolayer culture of mouse kidney. *Endocrinology.* 110: 1073–1075.

31. Trechsel, U., J-P. Bonjour, and H. Fleisch. 1979. Regulation of the metabolism of 25-hydroxyvitamin D_3 in primary cultures of chick kidney cells. *J. Clin. Invest.* 64:206–217.

32. Bikle, D. D., and H. Rasmussen. 1974. A comparison of the metabolism of 25-hydroxyvitamin D_3 by chick renal tubules, homogenates, and mitochondria. *Biochim. Biophys. Acta.* 362:439–447.

33. Bikle, D. D., and H. Rasmussen. 1975. The ionic control of 1,25-dihydroxyvitamin D_3 production by chick renal tubules. *J. Clin. Invest.* 55:292–298.

34. Rasmussen, H., M. Wong, D. D. Bikle, and D. B. P. Goodman. 1972. Hormonal control of the renal conversion of 25-dihydroxycholecalciferol to 1,25-dihydroxycholecalciferol. *J. Clin. Invest.* 51:2502–2504.

35. Rost, C. R., D. D. Bikle, and R. A. Kaplan. 1981. In vitro stimulation of 25-hydroxycholecalciferol 1α-hydroxylation by parathyroid hormone in chick kidney slices: evidence for a role for adenosine 3'5' monophosphate. *Endocrinology.* 108:1002–1006.

36. Henry, H. L. 1982. The role of parathyroid hormone in the regulation of the metabolism of 25-hydroxyvitamin D. *Mineral Electrolyte Metab.* 8:179–187.

37. Spanos, E., D. D. Barrett, K. T. Chong, and I. MacIntyre. 1978. Effect of oestrogen and 1,25-dihydroxycholecalciferol metabolism in primary chick kidney cell cultures. *Biochem. J.* 174:231–236.

38. Henry, H. L. 1979. Response of chick kidney cell cultures to 1,25 dihydroxyvitamin D_3. *In* Vitamin D. Basic Research and its Clinical Application. A. W. Norman, K. Schaefer, Dv. Herrath, H-G. Grigoleit, J. W. Coburn, H. DeLuca, E. B. Mawer, and T. Suda, editors. Walter de Gruyter, New York. 467–474.