

Identification and Regulation of 1,25-Dihydroxyvitamin D₃ Receptor Activity and Biosynthesis of 1,25-Dihydroxyvitamin D₃

Studies in Cultured Bovine Aortic Endothelial Cells and Human Dermal Capillaries

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Abstract

Because 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) has been shown to play roles in both proliferation and differentiation of novel target cells, the potential expression of 1,25(OH)₂D₃ receptor (VDR) activity was investigated in cultured bovine aortic endothelial cells (BAEC). Receptor binding assays performed on nuclear extracts of BAEC revealed a single class of specific, high-affinity VDR that displayed a 4.5-fold increase in maximal ligand binding (N_{\max}) in rapidly proliferating BAEC compared with confluent, density-arrested cells. When confluent BAEC were incubated with activators of protein kinase C (PKC), N_{\max} increased 2.5-fold within 6–24 h and this upregulation was prevented by sphingosine, an inhibitor of PKC, as well as by actinomycin D or cycloheximide. Immunohistochemical visualization using a specific MAb disclosed nuclear localized VDR in venular and capillary endothelial cells of human skin biopsies, documenting the expression of VDR, *in vivo*, and validating the BAEC model. Finally, additional experiments indicated that BAEC formed the 1,25(OH)₂D₃ hormonal metabolite from 25(OH)D₃ substrate, *in vitro*, and growth curves of BAEC maintained in the presence of 10⁻⁸ M 1,25(OH)₂D₃ showed a 36% decrease in saturation density. These data provide evidence for the presence of a vitamin D microendocrine system in endothelial cells, consisting of the VDR and a 1 α -hydroxylase enzyme capable of producing 1,25(OH)₂D₃. That both components of this system are coordinately regulated, and that BAEC respond to the 1,25(OH)₂D₃ hormone by modulating growth kinetics, suggests the existence of a vitamin D autocrine loop in endothelium that may play a role in the development and/or functions of this pathophysiologically significant cell population.

Introduction

It is well established that 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃)¹ is a crucial hormone in Ca²⁺ homeostasis (1).

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1. *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cells; DiC₈, sn-1,2-dioctanoylglycerol; MAb 9A7 γ 2b, MAb against the

Most, if not all, of the biological actions of 1,25(OH)₂D₃ are believed to be mediated by a high-affinity nuclear receptor (VDR) for the vitamin D hormone (2). Recent studies of cultured human promyelocytic leukemia cells (3), cultured human macrophages (4, 5), skin cells (6, 7) and other cells (8–12) have disclosed several new biological functions of 1,25(OH)₂D₃. These studies indicate that 1,25(OH)₂D₃ plays potential roles in cell proliferation/differentiation (13) and also is biosynthesized in several of its peripheral target cells (4–6) in addition to the traditional renal site of formation.

Because endothelial cells are a dynamic tissue with spontaneous or injury-dependent cell renewal and expression of specific cell functions at the blood/vessel-wall interface, these cells were examined to determine whether they are potential targets for 1,25(OH)₂D₃. Initially, the possible presence of specific binding sites for 1,25(OH)₂D₃ was probed using cultured bovine aortic endothelial cells (BAEC) as a model. When receptors for 1,25(OH)₂D₃ were observed, the following hypotheses were tested: (a) that the growth state of BAEC may be associated with changes in VDR activity; (b) that BAEC differentiation induced by activators of protein kinase C (PKC) (14–17) may be associated with VDR regulation; (c) that growth parameters of BAEC may be altered in response to 1,25(OH)₂D₃; (d) that the receptor may be expressed *in vivo* in endothelial cells in venules and capillaries of human skin; and (e) that BAEC may possess 1 α -hydroxylase activity to form the sterol hormone ligand for the receptor. These studies describe the results obtained in examining these possibilities and the data support the conclusion that endothelial cells are a target site for both 1,25(OH)₂D₃ production and receptor-mediated action.

Methods

Materials

1,25(OH)₂[26,27-methyl-³H]cholecalciferol (158 Ci/mmol) was obtained from Amersham Buchler (Braunschweig, FRG); unlabeled 1,25(OH)₂D₃ and 25(OH)D₃ were gifts from Dr. Calcanis (Hoffmann-La Roche, Grenzach, FRG); 1 α -(OH)D₃ and 24R,25(OH)₂D₃ (repimer of 24,25(OH)₂D₃) were obtained from the Duphar Company (Amsterdam, The Netherlands); ¹⁴C-methylated ovalbumin (20 μ Ci/mg protein), ¹⁴C-methylated globulin (25 μ Ci/mg protein), [³H]L amino acids (40 Ci/mmol), [³H]uridine (25 Ci/mmol), and [³H]thymidine (40 Ci/mmol) were purchased from New England Nuclear (Dreieich, FRG); hydroxyapatite, dithiothreitol, Triton X-100, sphingosine, 12-O-tetradecanoyl-13-acetate (TPA), α -4-phorbol-12,13-didecanoate (α -PDD), actinomycin D, cycloheximide, and BSA were obtained from Sigma Chemical Co. (Munich, FRG); DME, Dulbecco's PBS, fetal bovine serum, trypsin-EDTA, streptomycin, and penicillin were

1,25(OH)₂D₃ receptor; α -PDD, α -4-phorbol-12,13-didecanoate; PKC, protein kinase C; RP reverse phase; TPA, 12-O-tetradecanoylphorbol-13-acetate; VDR, 1,25(OH)₂D₃ receptor.

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obtained from Gibco Laboratories (Grand Island, NY). *sn*-1,2-Dioctanoylglycerol (DiC₈) was a gift from Dr. W. Sorg (German Cancer Research Center, Heidelberg, FRG).

Cell culture

BAEC were a gift from Dr. C. M. Gajdusek, (University of Washington at Seattle, Department of Pathology) and were characterized as described by Schwartz (18). Cells were cultured in 35- and 100-mm plastic dishes (Falcon Labware, Oxnard, CA) in DME containing 4.5 g/liter glucose and supplemented with 100 U/liter penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS) unless otherwise noted. Media were changed twice each week unless otherwise noted. Density-arrested cells attained a saturation density of $1.2\text{--}1.5 \times 10^7$ cells/100-mm dish ~ 5 d after reaching confluence. Cells were counted using a Rosenberg chamber and used for experiments between passages 3–12. More than 95% of the cells excluded trypan blue under all experimental conditions.

Incubation conditions

When VDR activity was to be determined in confluent, density-arrested cells, cells were maintained in medium containing 10% FBS for 5 d after reaching confluence. Before addition of TPA or DiC₈, the medium was removed and replaced with medium containing 0.5% FBS. TPA dissolved in acetone was added (10 µl) to give a final concentration of 10^{-8} M TPA and 0.01% acetone (or acetone alone as control). For studies with DiC₈, 10 ml of medium from the dish was removed and supplemented with DiC₈ dissolved in a concentrated solution of 10 µl acetone. The mixture was sonicated for 30 s on ice at low power output using a sonicator (Bandelin Sonorex RK 100; West Berlin, FRG). The medium was warmed to 37°C and added back to the cultures. Control cultures were treated identically but in the absence of DiC₈. Sphingosine was added 30 min before the addition of TPA by dissolving it in a concentrated solution of 50 µl PBS to give a final concentration of sphingosine of 10^{-8} M (or PBS as control).

Assays

Incorporation of [³H]thymidine into DNA, [³H]uridine into RNA, and [³H]L amino acids into protein were determined as radioactivity in TCA-precipitable material after incubation of cultures with 2 µCi/ml [³H]thymidine, 2 µCi/ml [³H]uridine, or 5 µCi/ml [³H]L amino acids, respectively, for 60 min. 1,25(OH)₂D₃ was measured by RIA as described elsewhere (19).

Assay of VDR activity

Nuclear preparation and extraction. Cell suspensions (1.5×10^7 cells/ml) obtained by trypsinization were homogenized in TED buffer, i.e., 10 mM Tris HCl, 1.5 mM EDTA, 2 mM DTT, 10 mM sodium molybdate, pH 7.4. Crude fractions and nuclear extracts were prepared as described elsewhere (7, 10) and used for sucrose density-gradient analysis, binding studies, or DNA affinity chromatography.

KTED extracts. Cell extracts were prepared as described elsewhere (7, 10, 20). In brief, cell suspensions (1.5×10^7 cells/ml) were homogenized in 0.4 M KTED buffer (TED buffer supplemented with 0.4 M KCl). A purified fraction was prepared by centrifugation at 205,000 g for 30 min using a rotor (model Ti-50; Beckman Instruments, Fullerton, CA). The supernatant was used for binding studies.

Sucrose density-gradient centrifugation. Sucrose density-gradient analysis was performed as follows. 200 µl samples of nuclear extract were incubated with 1,25(OH)₂[³H]D₃ alone or together with a 200-fold molar excess of 1,25(OH)₂D₃, 25(OH)D₃, or 24R,25(OH)₂D₃ for 2 h. Subsequently, samples were layered on top of preequilibrated (for 2 h at 4°C) gradients and centrifuged at 255,000 g for 21 h (SW 60 rotor; Beckman Instruments). Seven-drop fractions were collected. Sedimentation coefficients were calculated using [¹⁴C]ovalbumin (3.7 S) and [¹⁴C]γ-globulin (7.3 S) as standards.

Scatchard analysis. Saturation analyses according to Scatchard

were carried out as described previously (7, 10). 100-µl aliquots (protein concentration 0.5–1.0 mg/ml) were incubated for 16 h at 4°C with increasing concentrations (0.1–5.0 nM) of 1,25(OH)₂[³H]D₃ alone or in the presence of a 100-fold molar excess of unlabeled 1,25(OH)₂D₃. Bound 1,25(OH)₂[³H]D₃ was determined using the hydroxyapatite assay (7).

DNA cellulose chromatography. DNA cellulose was prepared from polymerized calf thymus DNA (type I, Sigma Chemical Co., St. Louis, MO) and cellulose (CG-11; Whatman Instruments, Clifton, NJ) as described earlier (7, 10) and formed into small columns (2 ml). Nuclear extracts were incubated with 1,25(OH)₂[³H]D₃ in the absence and in the presence of a 200-fold molar excess of unlabeled 1,25(OH)₂D₃ and applied to the preequilibrated (2 h, 4°C, TED buffer) DNA cellulose columns. The columns were then washed with 3-column vol of TED and eluted with a linear KCl gradient (0.1–0.8 M).

Immunocytochemical visualization of VDR in vascular endothelial cells of human skin. A VDR-specific rat MAb 9A7γ2b that was raised against chicken intestinal VDR, and has been shown previously to react with high affinity to several mammalian forms of VDR (21, 22), was used in all experiments. The epitope recognized by this antibody is 100% conserved between the avian and mammalian forms of the receptor. This MAb is specific for VDR and does not cross-react with the glucocorticoid receptor or the estrogen receptor (21, 22). To examine whether VDR is expressed in nuclei of endothelial cells of human dermis, immunostaining was performed using the labeled avidin-biotin technique that has been successfully applied to VDR immunocytochemistry by Milde et al. (23). Skin biopsies (medial aspect of thigh) of human volunteers were studied. Cryostat sections were fixed and incubated with 9A7γ2b MAb at a final dilution of 1:1,000 and the detailed procedures employed were that of Milde et al. (23). In addition, double labeling experiments were performed to further characterize and localize the cells stained with MAb 9A7γ2b. Cryostat sections were sequentially incubated with MAb 9A7γ2b and with mouse MAb against collagen type IV. The binding of the latter was visualized by mouse-specific fluorescein isothiocyanate-labeled antibodies. Controls of immunocytochemical staining included the following: (a) cryostat sections of chicken intestine stained by MAb 9A7γ2b as a positive control; (b) human skin biopsy sections incubated with rat MAb of the same subclass as MAb 9A7γ2b but directed against an unrelated antigen, or against the estrogen receptor, or a polyclonal rat IgG at similar final concentrations to that used for MAb 9A7γ2b as negative controls.

Metabolism of 25(OH)D₃. The metabolism of 25(OH)D₃ was studied in BAEC in the log growth phase (1.4×10^6 cells/100-mm dish) or in growth inhibited confluent cells (1.2×10^7 cells/100-mm dish) supplemented with 2.0% FBS. Cells were incubated with 10^{-8} M unlabeled 25(OH)D₃ or 10^{-8} M 25(OH)[³H]D₃ (153 Ci/mmol) for 8–12 h according to the methods of Reichel et al. (4). At the end of the incubation, cells and medium were harvested and vitamin D metabolites were extracted into 20 ml acetonitrile and stored in liquid N₂. After addition of 5 ml 0.4 M K₂HPO₄ (pH 10.6), the extract was applied to a C₁₈ Sep-Pak cartridge (Waters Associates, Millipore Corp., Milford, MA) equilibrated with 5 ml methanol and 5 ml H₂O. Vitamin D₃ metabolites were subsequently eluted with acetonitrile. Samples were dried under N₂ and vitamin D₃ metabolites were separated by reverse-phase (RP) HPLC on ultrasphere ODS C-18 columns (10 mm × 250 mm, 10-µm spheres; Beckman Instruments, Inc., Munich, FRG) in acetonitrile/methanol/H₂O (70:10:20; vol/vol/vol) at a flow rate of 1 ml/min. The RP-HPLC instrument was equipped with a pump (model 8700), an autosampler (model 8780) a variable wave length detector (model 8480 UV; all obtained from Spectra Physics, San Jose, CA), and a fraction collector (model Frac-100; Pharmacia Fine Chemicals, Uppsala, Sweden). 0.5 ml fractions were collected and either analyzed for radioactivity or for presence of 1,25(OH)₂D₃ by RIA. Parallel vitamin D₃ metabolite samples were analyzed by straight-phase HPLC using P 102 columns (10 mm × 250 mm, 5 µm spheres) (Lichrosorb Hibar; Merck, Darmstadt, FRG) in a hexane/isopropanol solvent system (9:1; vol/vol) at a flow rate of 4 ml/min. 2-ml fractions were analyzed for radioactivity or by RIA.

Results

Evidence for the presence of $1,25(\text{OH})_2\text{D}_3$ VDR in cultured BAEC. To determine if a specific VDR exists in cultured BAEC, cells were maintained in the logarithmic phase of growth (see below) and nuclear extracts were prepared as described elsewhere (7, 10, 20). Extracts were subjected to sucrose density centrifugation and fractions were assayed for binding of radiolabeled $1,25(\text{OH})_2\text{D}_3$ in the absence or presence of a 200-fold excess of unlabeled hormone. $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ bound to a macromolecule sedimenting in the range of 3.2–3.5 S, consistent with the known sedimentation coefficient of mammalian VDR (Fig. 1). Excess unlabeled $1,25(\text{OH})_2\text{D}_3$ competed with radiolabeled $1,25(\text{OH})_2\text{D}_3$ for binding to the receptor, whereas unlabeled $25(\text{OH})\text{D}_3$ did not compete and $24\text{R},25(\text{OH})_2\text{D}_3$ competed only slightly. To further characterize the VDR macromolecule, nuclear extracts of BAEC were labeled with $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ and subjected to DNA cellulose chromatography as previously described (7). The VDR complex bound to DNA cellulose and eluted at 0.22 M KCl (Fig. 2), a characteristic feature of the steroid hormone receptor complex (3, 7, 9). Thus, BAEC possess a protein with the biochemical properties of the VDR.

Rapidly proliferating BAEC express increased VDR activity. To investigate whether expression of VDR depends on the growth state of the cells, BAEC were maintained either in the logarithmic phase of growth or as nongrowing confluent cells at saturation density (see below). The binding characteristics of radiolabeled $1,25(\text{OH})_2\text{D}_3$ to its receptor in growing and nongrowing cells were then compared. In each of three independent experiments, maximal binding (N_{max}) of $1,25(\text{OH})_2\text{D}_3$ to its receptor was 4.5-fold higher in rapidly proliferating BAEC compared with their confluent, density-arrested counterparts (Fig. 3). In contrast, the apparent affinity of the hormone for its receptor (K_d) remained unchanged (Fig. 3).

Activators of PKC cause upregulation of VDR. Activators of PKC have previously been shown to stimulate the expression of several biological activities in endothelial cells (14, 15,

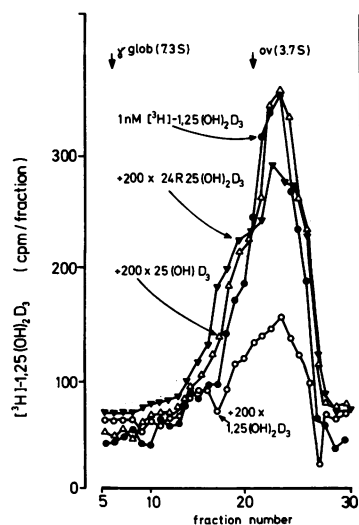


Figure 1. Sucrose density-gradient analysis of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ binding to the nuclear fraction of BAEC. A nuclear extract from 10^7 cells in logarithmic growth phase ($1.4 \times 10^5/\text{cm}^2$) was prepared in KTED buffer (0.5 mg protein/ml) and incubated for 2 h at 4°C with 1 nM $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ alone or along with 200-fold molar excess of the unlabeled vitamin D metabolites as indicated in the figure. Sedimentation was performed in linear 5–20% sucrose density gradients by ultracentrifugation (255,000 g for 21 h at 4°C). Arrows indicate $[^{14}\text{C}]$ ovalbumin (3.7 S) and $[^{14}\text{C}]$ bovine- γ -globulin (7.3 S).

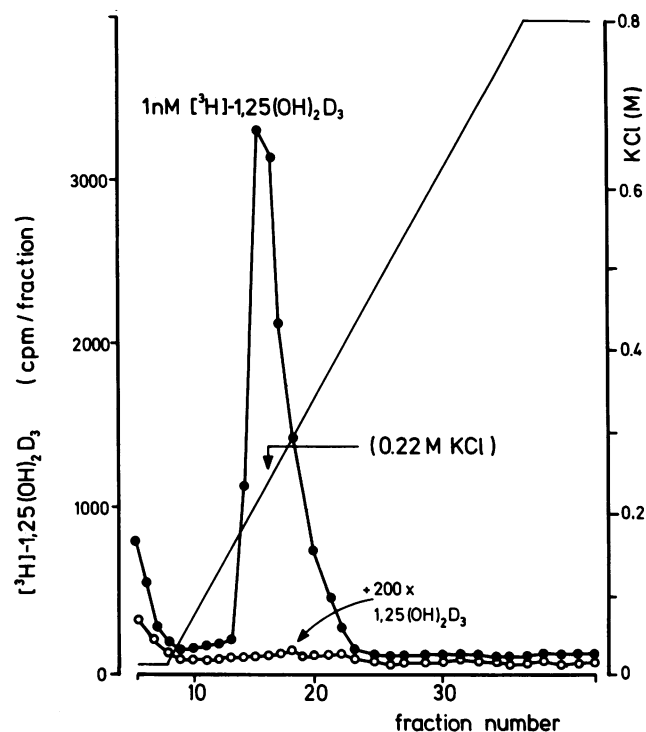


Figure 2. Elution of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ -labeled receptor from a DNA cellulose column. Nuclear fractions of 10^7 BAEC in logarithmic growth phase ($1.4 \times 10^5/\text{cm}^2$) were preincubated with 1 nM $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ alone, or with 200-fold molar excess of unlabeled $1,25(\text{OH})_2\text{D}_3$, and applied to a 1.5×6 cm column of DNA cellulose. After extensive washing, the column was eluted with a linear gradient of 10 vol of 0.1–0.8 M KCl.

24) and to have profound effects on endothelial cell functions (16). VDR activity was therefore examined to determine if it is altered in BAEC stimulated by activators of PKC. Incubation of confluent BAEC with 10^{-8} M TPA resulted in a 2.5-fold increase in VDR number without a change in the K_d value (Fig. 4 A). This stimulatory effect of TPA became apparent

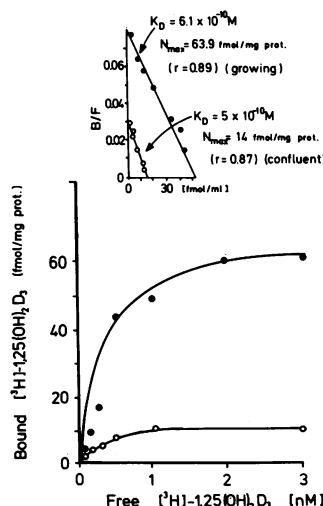


Figure 3. Influence of BAEC cell growth on nuclear $1,25(\text{OH})_2\text{D}_3$ receptor expression. Growing (1.4×10^5 cells/ cm^2 ; ●) and confluent (6.1×10^5 cells/ cm^2 ; ○) cells were used. Nuclear extracts (0.6 mg protein/ml) were incubated with increasing concentrations (0.1–3 nM) of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ in the presence or absence of a 100-fold molar excess of unlabeled $1,25(\text{OH})_2\text{D}_3$ for 16 h at 4°C . Bound and free $1,25(\text{OH})_2\text{D}_3$ were separated with hydroxyapatite. The saturation curve (bottom) shows specific binding with a plateau occurring at 2–3 nM. The equilibrium dissociation constants, K_d , were calculated from the slope of the regression lines, as depicted in the Scatchard plots (top); N_{max} was determined by extrapolation of the lines to the abscissa.

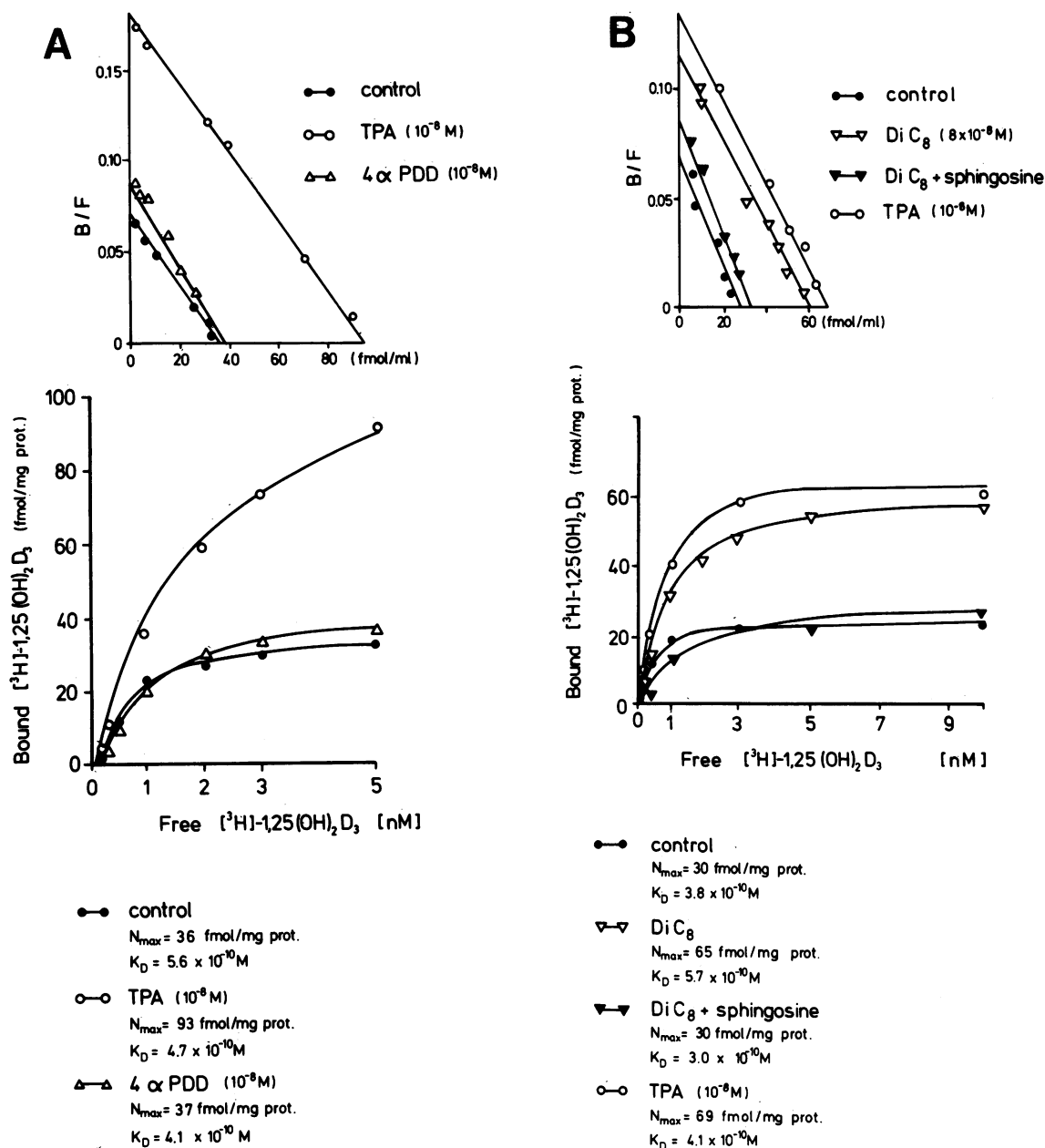


Figure 4. Effects of TPA, 4 α -PDD, DiC₈, and sphingosine on specific binding of 1,25(OH)₂[³H]D₃ in KTED extracts of confluent BAEC. In A, B, and C, the lower illustration is the saturation curve and the upper graph is the Scatchard analysis of the data. N_{max} and K_D are listed below in each case. (A) BAEC were first grown to confluence in 100-mm plates containing 30 ml of DME with 10% FBS. On day five, density-dependent, growth-inhibited cells (1.5×10^7 cells/100-mm dish) were fed with medium containing 0.5% FBS and divided into three groups: solvent control (30 μ l acetone), 10^{-8} M TPA, or 10^{-8} M 4 α -PDD. Incubation was continued for 24 h. (B) BAEC were grown as described above and divided into four groups: control (30 μ l acetone), 10^{-8} M TPA, 8×10^{-8} M DiC₈, or 8×10^{-8} M DiC₈ in the presence of 5×10^{-6} M sphingosine. Incubation was for 24 h. (C) BAEC were grown as described above and four groups were examined: control (30 μ l acetone), 10^{-8} M TPA, 5×10^{-6} M sphingosine, or 10^{-8} M TPA plus 5×10^{-6} M sphingosine. Incubation was for 24 h.

within 6 h and increased over the next 18 h (data not shown). To assess the effects of TPA on the growth rate of BAEC, the rate of [³H]thymidine incorporation into DNA was measured. Confluent, density-arrested BAEC had low but significant rates of DNA synthesis when compared with rapidly proliferating cells, presumably reflecting a low cell turnover in confluent cultures (data not shown). However, this basal low rate of DNA synthesis was further suppressed by 55% in the presence of TPA. This negative effect of TPA on DNA synthesis

was detectable within 2 h (data not shown) and indicates that TPA does not enhance VDR levels by stimulating BAEC proliferation. Conversely, and as has been observed by others (16), TPA treatment of BAEC resulted in extensive cell sprouting. α -PPD, a phorbol ester that is unable to stimulate PKC in several biological systems (25), did not induce upregulation of VDR in BAEC (Fig. 4 A). α -PDD was also unable to induce morphological changes in BAEC. To investigate further the involvement of PKC in the upregulation of VDR, confluent

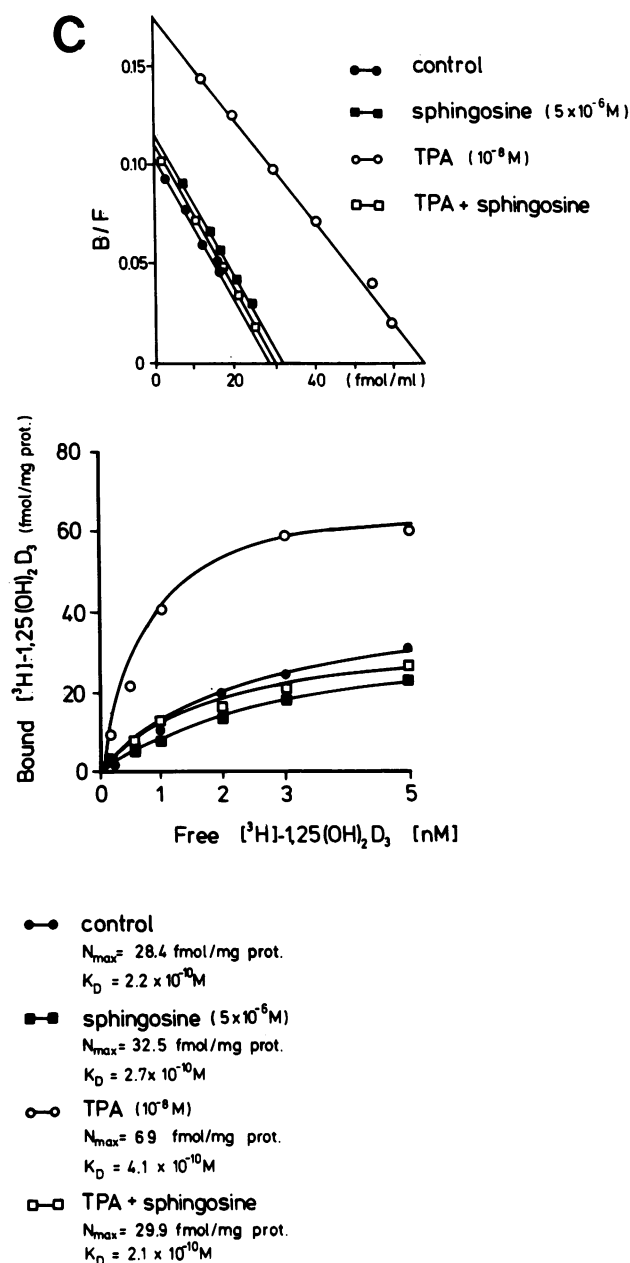


Figure 4 (Continued)

BAEC were incubated with DiC_8 , another potent activator of PKC (25, 26). Treatment of BAEC with DiC_8 resulted in an upregulation of VDR activity similar to that elicited by TPA (Fig. 4 B). Sphingosine, a recently described inhibitor of PKC (27), prevented both TPA-dependent (Fig. 4 C) and DiC_8 -dependent (Fig. 4 B) upregulation of VDR activity.

PKC-dependent upregulation of VDR requires transcription and translation. To determine whether PKC-dependent upregulation of VDR activity required transcriptional activity, confluent, density-arrested BAEC were incubated with 2 $\mu\text{g/ml}$ actinomycin D. Whereas TPA by itself had no significant effect on total RNA synthesis, 2 $\mu\text{g/ml}$ actinomycin D blocked the rate of uridine incorporation into RNA by > 85% without compromising the viability of the cells within 24 h (data not shown). Actinomycin D prevented TPA-dependent

Table I. Actinomycin D and Cycloheximide Prevent TPA-dependent Upregulation of VDR in Growth-inhibited, Density-arrested Endothelial Cells*

| VDR (fmol/mg protein) | | | |
|---------------------------------------|----|---------------------------------------|----|
| Experiment A | | Experiment B | |
| Control | 30 | Control | 30 |
| + TPA (10^{-8} M) | 73 | + TPA (10^{-8} M) | 96 |
| + Actinomycin D (2 $\mu\text{g/ml}$) | 31 | + Cycloheximide (5 $\mu\text{g/ml}$) | 28 |
| + TPA (10^{-8} M) | 37 | + TPA (10^{-8} M) | 35 |
| + Actinomycin D (2 $\mu\text{g/ml}$) | | + cycloheximide (5 $\mu\text{g/ml}$) | |

1.2×10^7 cells/100-mm dish.

induction of VDR activity (Table I). Similarly, TPA had no significant effect on the overall rates of amino acid incorporation into protein. 5 $\mu\text{g/ml}$ cycloheximide blocked the rate of labeled amino acid incorporation into protein by > 85% while it did not compromise the viability of the cells within 24 h (data not shown). Cycloheximide completely prevented TPA-dependent upregulation of VDR (Table I).

1,25(OH) $_2$ D $_3$ affects the growth of BAEC. To study the effects of 1,25(OH) $_2$ D $_3$ on BAEC growth, cell number and the rate of [^3H]thymidine incorporation into DNA were monitored in the presence and absence of 10^{-8} M 1,25(OH) $_2$ D $_3$. BAEC treated with 1,25(OH) $_2$ D $_3$ attained a significantly lower saturation density compared with cells maintained in the absence of 1,25(OH) $_2$ D $_3$ (Fig. 5). This effect of the hormone was accompanied by a significant decrease in the rate of [^3H]thymidine incorporation into DNA (Table II). The data in Table II also show that the relative potency of the various vitamin D metabolites in inhibiting thymidine incorporation parallels their affinities for the VDR as previously shown (7) and illustrated in part by the competition results in Fig. 1. Therefore 1,25(OH) $_2$ D $_3$ appears to influence the growth of BAEC via its binding to the high-affinity nuclear VDR.

Immunohistochemical evidence of in vivo expression of VDR in endothelial cells of venules and capillaries of human skin. The potential in vivo expression of VDR in endothelial cells was studied by examining human tissue, namely dermal vessels in sections of skin biopsies. The reactivity of the nuclei in endothelial cells of dermal blood vessels with antireceptor MAb (MAb 9A7 γ 2b) is demonstrated in Fig. 6 B and D. Double-staining experiments were carried out with mouse anticollagen type IV MAb to visualize the basement membrane of the vessels in the same section. (Fig. 6, A and C). These studies showed that immunoreactive VDR is present in the nuclei of cells within the basement membrane; the topographical relationship to the basement membrane identifies these cells as endothelial cells. To ascertain the specificity of these findings, several control experiments were performed. When applied to chicken intestinal tissue as a positive control, the anti-VDR antibody decorated the nuclei of the absorptive epithelial cells that are known to localize radiolabeled 1,25(OH) $_2$ D $_3$ (Fig. 6 E). The high sensitivity of this immunocytochemical technique was demonstrated by the observation that MAb 9A7 γ 2b reacted to chick intestinal nuclei at dilutions as low as 1:64,000. Under the blocking conditions used, nonspecific binding of the streptavidin enzyme complex was minimal. Peroxidase-positive macrophages (arrows) in intestine could

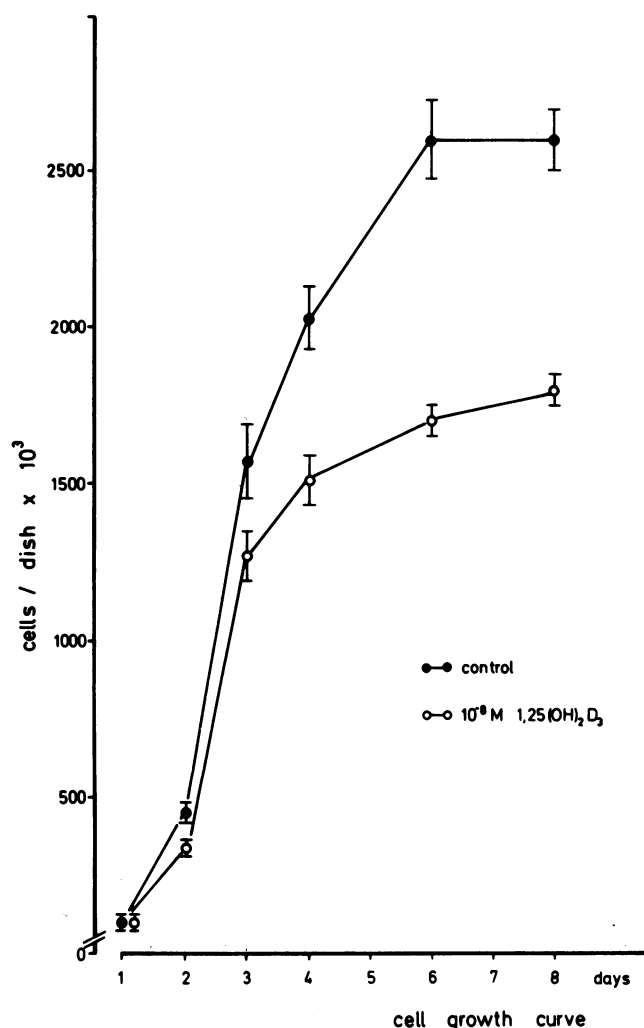


Figure 5. Action of 1,25(OH)₂D₃ on growth parameters of BAEC. Cells were plated at a density of 8.8×10^4 cells per 35-mm dish and cultured in DME with 10% FBS; medium was changed daily. 1,25(OH)₂D₃ at a final concentration of 10^{-8} M or ethanol (0.02%) as solvent control were added. The data represent the mean of three dishes \pm SD.

be distinguished easily from other nuclear stained cells because of their heavy cytoplasmic staining. Also, chick intestinal nuclei were not stained by polyclonal rat IgG (Fig. 6 F). Furthermore, rat MAb against the estrogen receptor showed no reaction with epithelial cells of chick intestine, whereas heavy staining was observed in smooth muscle cell nuclei of human uterus (data not shown). When a separate rat MAb against an unrelated antigen or polyclonal rat IgG was used, no reactivity with endothelial cells nuclei was observed (data not shown).

BAEC synthesize 1,25(OH)₂D₃ from 25(OH)D₃. To test the ability of BAEC to form the active hormone, BAEC were examined under two growth conditions, i.e., confluent, density-arrested and growing (log phase) cells. Parallel cultures were incubated in the presence of radiolabeled 25(OH)D₃ according to the procedure of Reichel et al. (4). Transformation of 10^{-6} – 10^{-11} M 25(OH)[³H]D₃ was examined in BAEC in the log growth phase (1.4×10^6 cells/100-mm dish) or in growth inhibited confluent cells (1.2×10^7 cells/100-mm dish) in culture medium supplemented with 2% FBS. No transformation

Table II. Dose Response of [³H]Thymidine Incorporation into BAEC in Log Phase of Growth*

| Vitamin D metabolite added | [³ H]Thymidine incorporation % of solvent control \pm SD |
|--|---|
| Solvent control | 100 \pm 6.9* |
| 10^{-11} M 1,25(OH) ₂ D ₃ | 100 \pm 5.8 |
| 10^{-10} M 1,25(OH) ₂ D ₃ | 87 \pm 1.0 |
| 10^{-9} M 1,25(OH) ₂ D ₃ | 78 \pm 7.8 |
| 10^{-8} M 1,25(OH) ₂ D ₃ | 33 \pm 0.2 |
| 10^{-8} M vitamin D ₃ | 90 \pm 7.0 |
| 10^{-8} M 25(OH)D ₃ | 86 \pm 3.1 |
| 10^{-8} M 24R,25(OH) ₂ D ₃ | 81 \pm 1.3 |
| 10^{-8} M 1 α (OH)D ₃ | 71 \pm 1.3 |

Cells were plated at a density of 1.2×10^5 cells/35-mm dish and grown in DME with 10% FBS. On day 2, the medium was changed and 1,25(OH)₂D₃ (final concentration 10^{-11} – 10^{-8} M), other metabolites of vitamin D₃, or solvent (0.02% ethanol) were added. 16 h after the medium change, 2.5 μ Ci [³H]thymidine/dish were added. The data represent the mean of three dishes.

* 100% is [³H]thymidine incorporation in control dishes (range 33,900 to 35,600 cpm/dish).

of 25(OH)[³H]D₃ was observed after incubation in media alone (in the absence of cells) for 8 h. When growing BAEC were incubated with 25(OH)[³H]D₃ for 8–12 h, there was conversion of 25(OH)[³H]D₃ to a metabolite that comigrated with unlabeled or radioactive 1,25(OH)₂D₃ in both straight-phase (Fig. 7 A) or RP-HPLC (Fig. 7 B) systems. RIA of 1,25(OH)₂D₃ applied to aliquots of the HPLC fractions confirmed the presence of 1,25(OH)₂D₃. As depicted in Fig. 8, the metabolism of 25(OH)D₃ to 1,25(OH)₂D₃ was substrate concentration dependent, with half-maximal conversion occurring between 10^{-9} and 10^{-8} M substrate. It is noteworthy that there was little production of 1,25(OH)₂D₃ from 25(OH)D₃ by confluent cells (Fig. 8), suggesting that the capacity to form 1,25(OH)₂D₃ is greatly enhanced in rapidly growing cells as is the 1,25(OH)₂D₃ receptor number.

Discussion

The findings of this study can be summarized as follows: (a) BAEC express a VDR-like macromolecule that is indistinguishable from the classical VDR originally described in chicken intestinal mucosa and subsequently demonstrated in a number of mammalian cells (2); (b) maintenance of BAEC at low cell density to induce cell growth upregulates the expression of VDR; (c) density-arrested BAEC can be stimulated to express VDR by activators of PKC; (d) BAEC respond to the addition of exogenous 1,25(OH)₂D₃ hormone by a decrease in their growth rate and saturation density; (e) VDR is expressed in both capillary and venule endothelial cells of normal human skin, in vivo; (f) rapidly proliferating BAEC, but not density-arrested BAEC, metabolize 25(OH)D₃ to a sterol with chromatographic and immunoreactive properties identical to those of 1,25(OH)₂D₃. These results represent the first demonstration of a steroid hormone receptor in endothelial cells and reveal two potential triggers for the upregulation of the VDR, namely increased proliferation and activation of PKC. A fur-

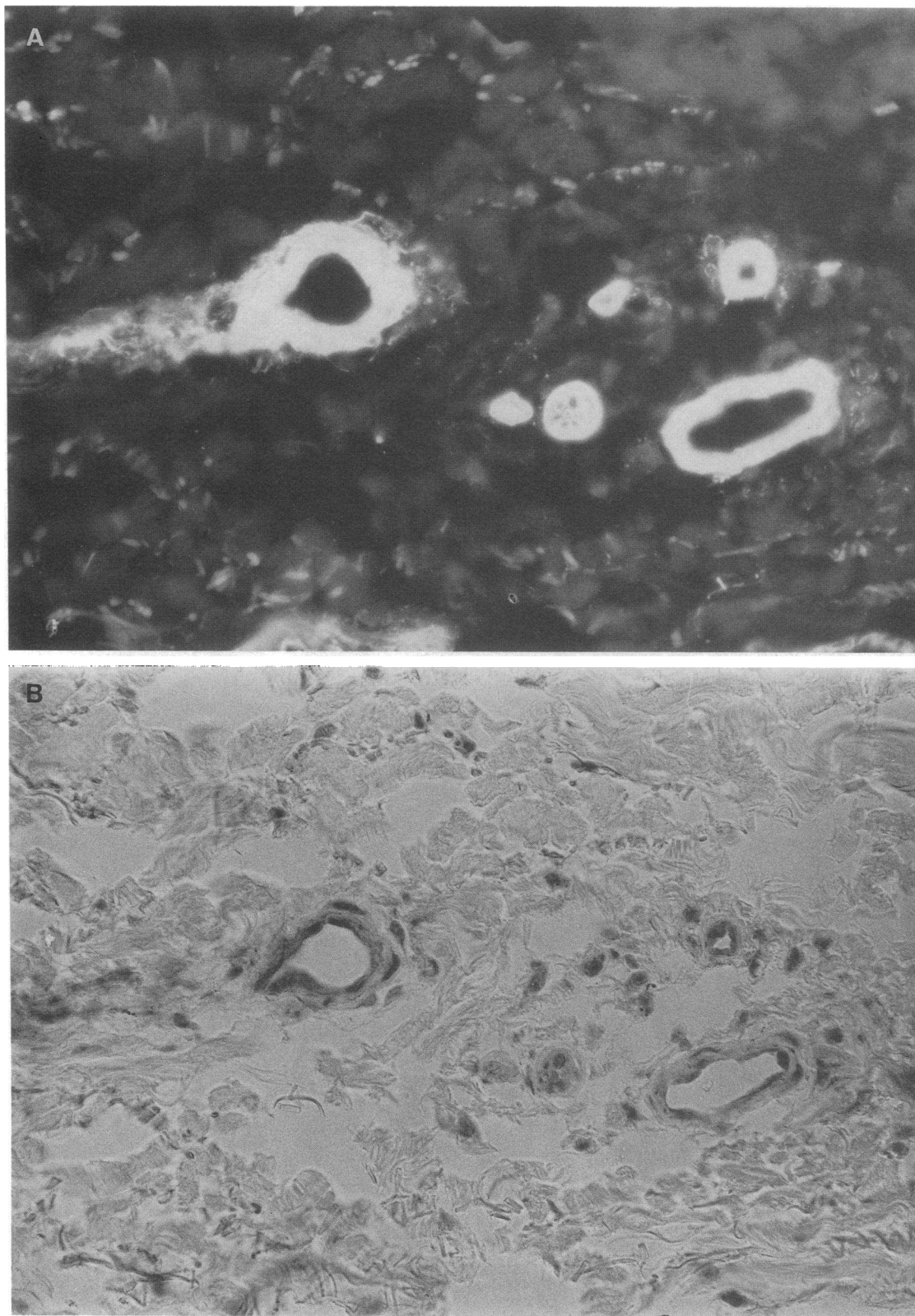


Figure 6. Double immunohistochemical staining of normal human skin with anticollagen type IV and anti-VDR MAb and reaction of anti-VDR antibody with chicken intestinal mucosa nuclei as a positive control. (A, C) Staining of normal human skin (×450) for collagen type IV by indirect immunofluorescence. (B, D) Staining for VDR by the labeled avidin-biotin technique (23) using a specific MAb (9A7γ2b). Note that the positively stained and elongated nuclei of endothelial cells are located within the areas illuminated by anticollagen type IV in a and c. (E) Demonstration of VDR in chicken intestinal nuclei (×950) with MAb 9A7γ2b. (F) Specificity control of chicken intestine with a dilution of rat IgG replacing the MAb 9A7γ2b used in E. In this negative control, peroxidase-positive macrophages are evident (arrows), but staining of epithelial cell nuclei is absent.

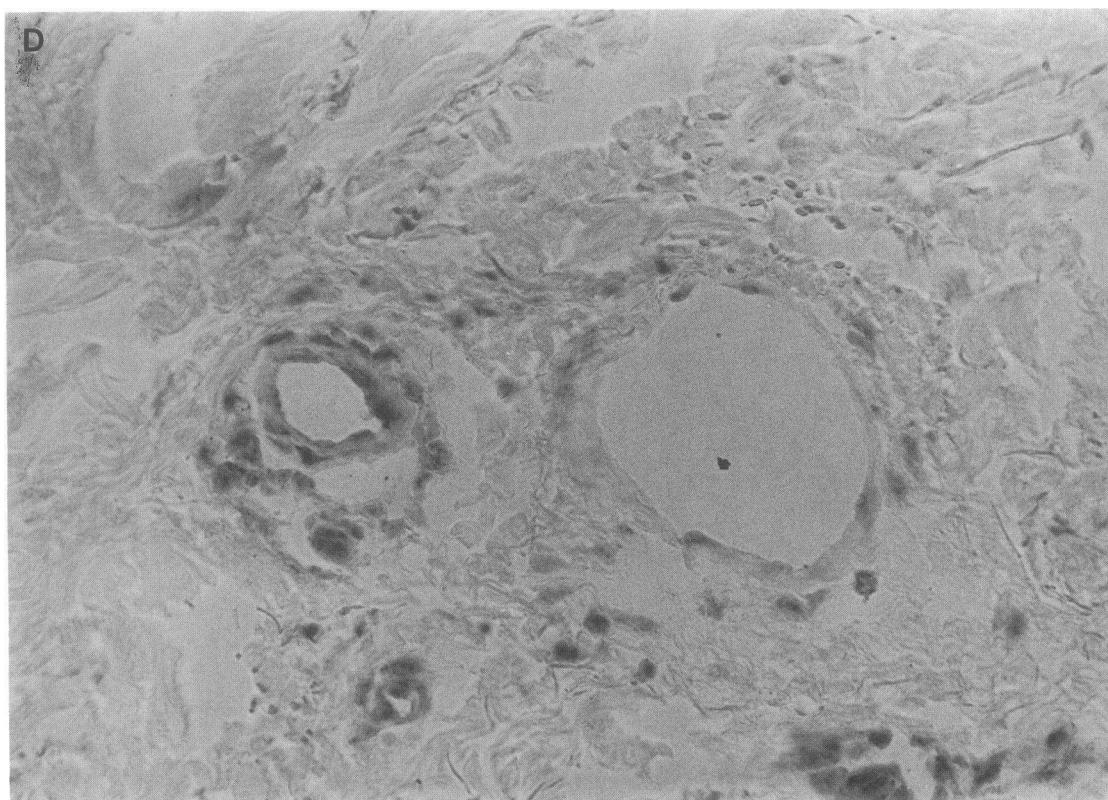
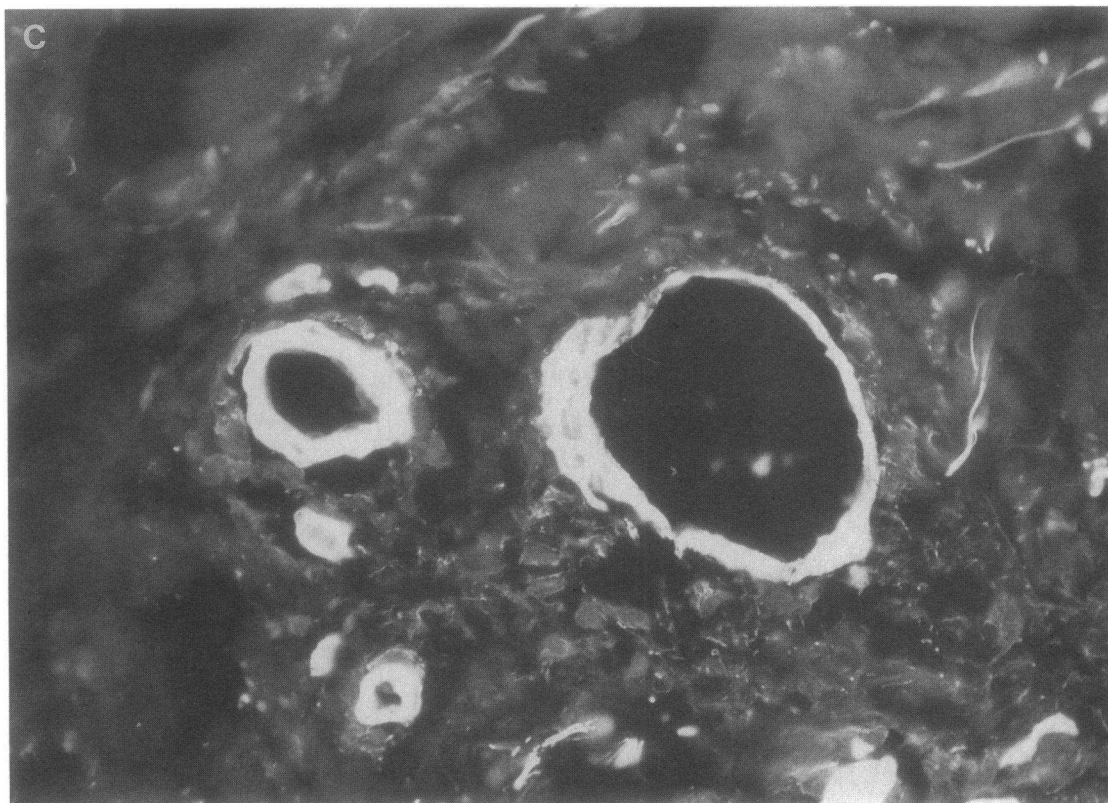


Figure 6 (Continued)

ther aspect of the results is that rapidly proliferating BAEC both synthesize $1,25(\text{OH})_2\text{D}_3$ and decrease their growth rate in response to the hormone, suggesting that a function of

$1,25(\text{OH})_2\text{D}_3$ in endothelial cells could be to modulate proliferation via an autocrine feedback mechanism.

Several lines of evidence document the presence of specific

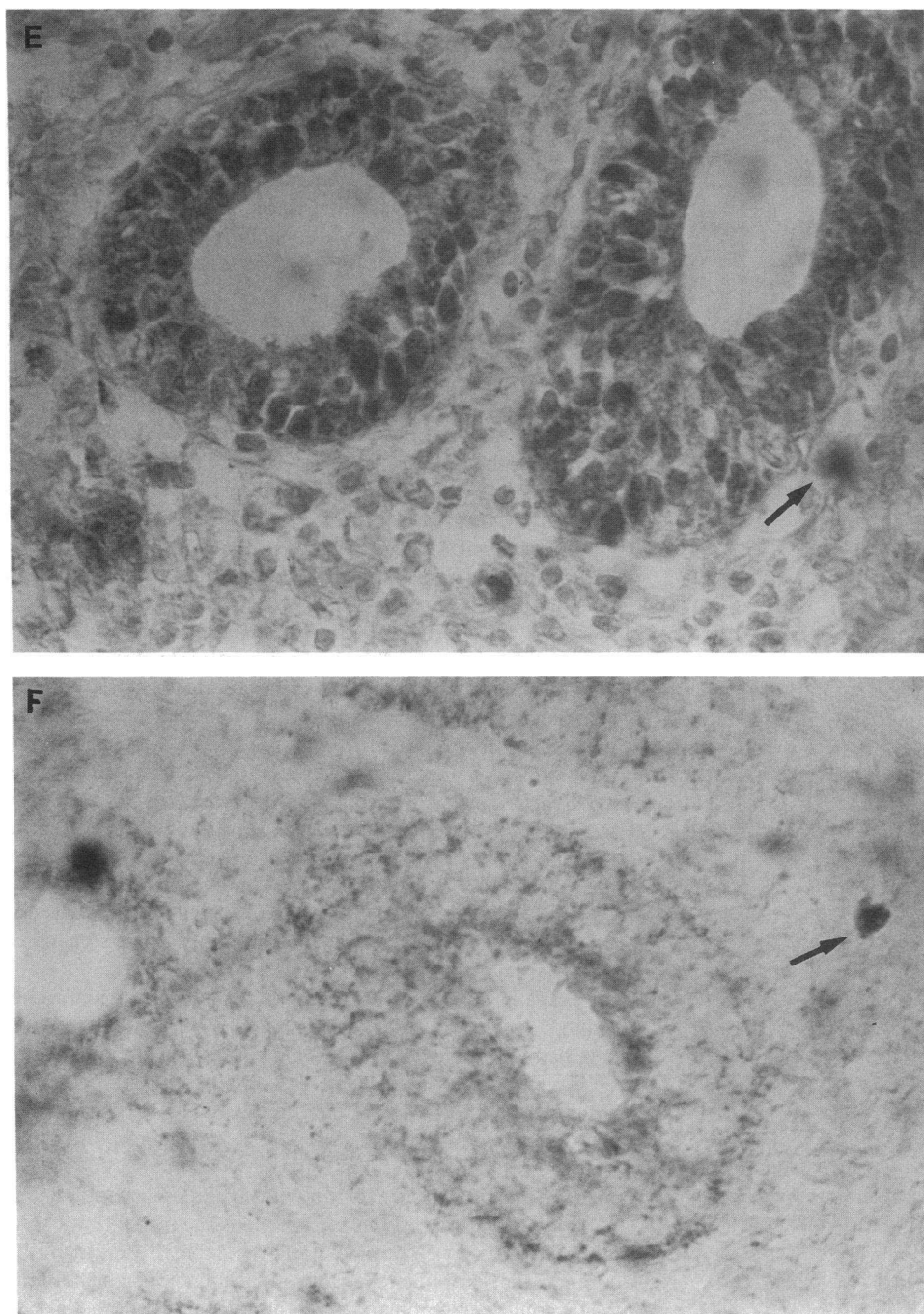


Figure 6 (Continued)

VDR in cultured BAEC. For instance, radiolabeled $1,25(\text{OH})_2\text{D}_3$ specifically binds to the nuclear fraction of BAEC and exhibits the characteristic VDR sedimentation coefficient of 3.2–3.5 S (Fig. 1). Moreover, the nuclear receptor interacts with DNA cellulose and the active component can be eluted at a concentration of 0.22 M KCl (Fig. 2). Scatchard analyses indicate the presence of a single receptor class with a K_d of 5×10^{-10} M (Fig. 3). These biochemical properties of the VDR-like activity in BAEC are consistent with the presence of a molecule with two binding domains, one for DNA and another for the hormone. Thus, this activity in BAEC is distinct

from the previously described serum vitamin D binding protein (28) but shares all characteristics of the intracellular VDR of chicken intestinal mucosa (2, 22) and other tissues (3, 7, 10, 11, 12, 29). It is therefore concluded that BAEC possess the specific VDR.

Having identified the VDR in BAEC, it was next sought to determine whether expression of the receptor can be modulated. One of the characteristics of endothelial cells is their ability to form a confluent, density-arrested monolayer. A number of metabolic activities of endothelial cells are altered when this monolayer is disrupted and cell growth is induced by

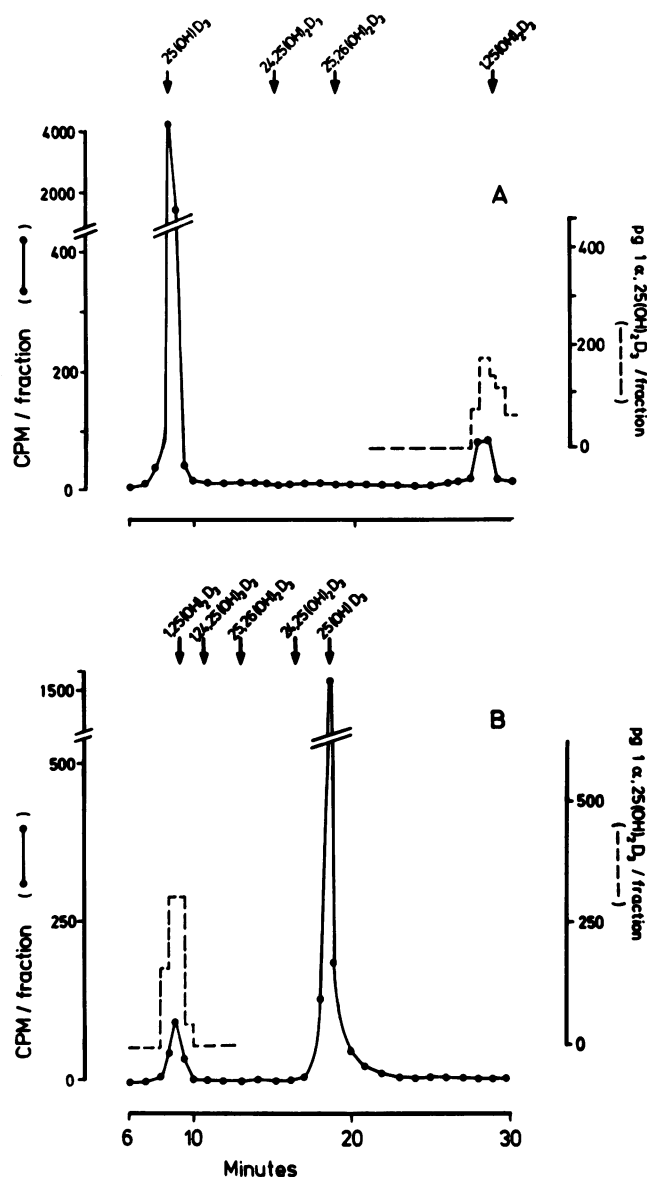


Figure 7. Metabolism of $25(\text{OH})\text{D}_3$ to $1,25(\text{OH})_2\text{D}_3$ by BAEC. BAEC were grown to a density of 1.4×10^6 cells/100 mm dish in 10 ml RPMI 1640 medium with 2% FBS. Incubation of the cells was carried out with 10^{-7} M $25(\text{OH})[^3\text{H}]\text{D}_3$ (153 Ci/mmol) or unlabeled $25(\text{OH})\text{D}_3$ for 8–12 h. Cells and medium were extracted (see Methods) and the extract was applied directly to a prewashed (5 ml methanol, 5 ml H_2O) C_{18} Sep-Pak cartridge and the vitamin D metabolites eluted with acetonitrile. The metabolites then were resolved on two HPLC systems. (A) Straight-phase HPLC was performed using a Lichrosorb Hilar P 102 column (10 mm \times 250 mm \times 5- μm spheres) at a flow rate of 4 ml/min using an isocratic system of hexane-isopropanol (9:1). $\frac{1}{2}$ -min fractions (2 ml) were collected and fractions were counted (—) and aliquots (10 μl) evaluated by $1,25(\text{OH})_2\text{D}_3$ RIA to detect radioinert hormone (-----). (B) RP-HPLC was performed using an ultrasphere column (10 mm \times 250 mm \times 10- μm spheres), eluted at a flow rate of 1 ml/min with a gradient system of acetonitrile/methanol/water (70:10:20 vol/vol/vol). $\frac{1}{2}$ -min fractions (0.5 ml) were collected for 30 min. Counting and RIA were as in A.

seeding the cells at low cell density (18). In these experiments, nuclear extracts prepared from rapidly growing BAEC showed a major increase in the N_{max} value for VDR, but no change in

K_d when compared with their nongrowing, density-arrested counterparts. These results indicate that rapid growth of BAEC is associated with upregulation of the number of functional VDR molecules rather than with a change in the affinity of the hormone for its receptor. Increased VDR expression has been observed previously in rapidly proliferating cultured mouse bone cells (30) and osteogenic sarcoma cell lines treated with glucocorticoids (31) and retinoic acid (32).

Activation of PKC by TPA has been shown to have profound effects on BAEC. Endothelial cells treated with TPA display a more differentiated phenotype (33), including the formation of fenestrae (16) and capillary-like structures (15), synthesis of plasminogen activator and plasminogen activator inactivator (24), as well as several other morphological alterations (14). Therefore, TPA and the short chain diglyceride, DiC_8 (26), were used to activate PKC in BAEC. In density-arrested endothelial cells, VDR is dose-dependently increased by the two activators of PKC. Furthermore, VDR induction was blocked by the PKC inhibitor sphingosine. The finding of PKC-dependent VDR upregulation is analogous to previous observations of increased glucocorticoid receptor activity in myeloid leukemia cells treated with TPA (34). In that study, increased glucocorticoid receptor expression paralleled induction of differentiation of the leukemic cells into mature macrophages. It is of interest that the activators of PKC evoke changes in VDR expression similar to those caused by enhanced proliferation (compare Figs. 3 and 4). The mechanisms underlying this upregulation remain to be determined, but the results of experiments employing actinomycin D and cycloheximide (Table I) indicate that both transcriptional and translational activity are required. However, that TPA inhibits the rate of DNA synthesis in BAEC suggests that the intracellular pathways responsible for PKC activation of VDR expression are distinct, in part, from those occurring when BAEC are induced to grow rapidly.

Previous studies by Stumpf et al. (35, 36) using autoradiographic localization of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ in nuclei, *in vivo*, revealed the presence of VDR in a variety of cells and tissues. However, no specific evidence was obtained by this procedure for the presence of VDR in endothelial cells. The sensitivity of the autoradiographic localization technique may be lower than that used in this study. To examine whether the VDR is expressed in endothelial cells *in vivo*, venules, arterioles, and capillaries of normal skin were probed using high affinity MAb 9A7 γ 2b (22, 37), coupled with the labeled acidin-biotin method that amplifies the immunohistochemical signal (23). VDR could be demonstrated in the nuclei of endothelial cells of human dermal vessels (Fig. 6). By double immune staining, using anticollagen type IV antibodies that delineate the capillary basement membrane, endothelial cells could be distinguished from vascular smooth muscle cells, which also express VDR activity (11). Therefore, the immunocytochemical data support indirectly the biochemical observation of high-affinity binding of $1,25(\text{OH})_2\text{D}_3$ to VDR in cultured BAEC, and extend the finding by documenting that immunoreactive VDR is present in the nuclei of normal human endothelial cells, *in vivo*.

$1,25(\text{OH})_2\text{D}_3$ inhibits cell proliferation and induces differentiation in a variety of cell systems (2, 3, 6, 7, 9, 12) and, in this investigation, $1,25(\text{OH})_2\text{D}_3$ reduced thymidine incorporation and saturation density of BAEC (Table II and Fig. 5). The effect was specific, because the parent vitamin D_3 compound

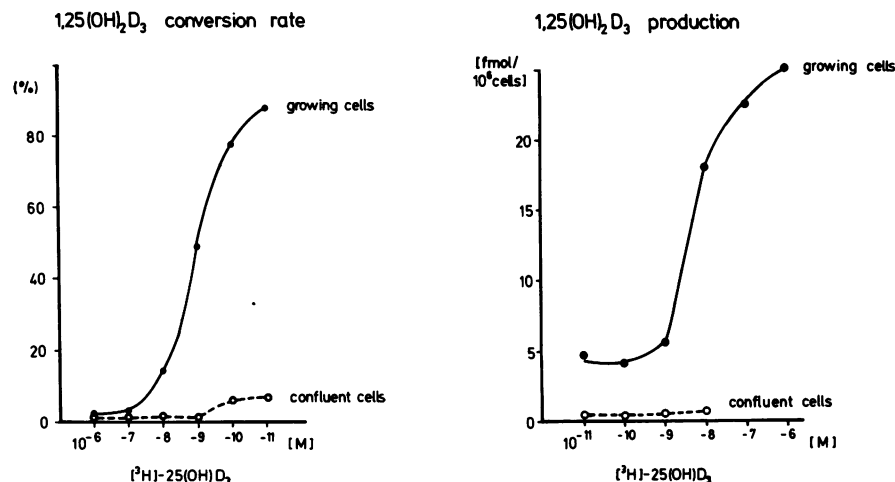


Figure 8. Dose-dependent biotransformation by BAEC of 25(OH)D₃ to a more polar metabolite comigrating with 1,25(OH)₂D₃ on RP-HPLC. BAEC were harvested in logarithmic growth (1.4×10^6 cell/100 mm dish) (—) or at a confluent density of 1.2×10^7 cells/100 mm dish (---) in 10 ml RPMI 1640 medium with 2% FBS. Incubation was performed with 10^{-11} – 10^{-6} M 25(OH)[³H]D₃ (153 Ci/mmol) for 8 h. Cells and medium were extracted (see Methods) and the extract was applied directly to a prewashed (5 ml methanol, 5 ml H₂O) C₁₈ Sep-Pak cartridge. RP HPLC was carried out on the Sep-Pak eluent with an ultrasphere (10 mm \times 250 mm \times 10- μ m spheres) column developed at a flow rate of 1 ml/min with gradient system of acetonitrile/methanol/water (70:10:20). $\frac{1}{2}$ -min fractions (0.5 ml) were collected for 30 min. Data are expressed as percent conversion to 1,25(OH)₂D₃ (left) or femtomoles 1,25(OH)₂D₃/10⁶ cells (right).

exhibited no significant inhibitory action on thymidine incorporation. The observation of a small, but significant inhibition of DNA synthesis by 25(OH)D₃ and 24R,25(OH)₂D₃ (Table II) raised the possibility that 1 α -hydroxylase activity may exist in endothelial cells. Extrarenal 1 α -hydroxylase activity has been identified recently in several tissues (4–6, 8, 38, 39). The evidence for production of 1,25(OH)₂D₃ by BAEC reported herein includes the following: (a) the radiolabeled or unlabeled product comigrated with chemically synthesized 1,25(OH)₂D₃ standard in two different HPLC systems and (b) the product was recognized by a specific 1,25(OH)₂D₃ antibody. The putative 1 α -hydroxylase activity was biologically modulated in that it depended on cell growth phase and was demonstrable in growing, but not in confluent, growth-inhibited cells. Synthesis of 1,25(OH)₂D₃ was a function of the concentration of the 25(OH)D₃ substrate and half-maximal conversion occurred at a substrate level near the recognized K_m for the renal 1 α -hydroxylase. The amount of 1,25(OH)₂D₃ synthesized at 10^{-8} M 25(OH)D₃ by growing BAEC, namely 11 pg/10⁶ cells per 8 h, is low compared with the 25 ng/mg protein per h reported for primary cultures of chick kidney cells. Thus, the turnover number of the endothelial cell 1 α -hydroxylase is minimal, but local (free) 1,25(OH)₂D₃ concentrations in endothelial cells could conceivably exceed the 1,25(OH)₂D₃ concentrations generated in the circulation by the renal endocrine cells. We speculate that the endothelial cell comprises a vitamin D microendocrine system capable of autonomous regulation of cell function.

This study of VDR expression in, and 1,25(OH)₂D₃ generation by, BAEC raises new questions about the biology of the endothelium. First, what is the physiological significance of the VDR in endothelial cells? The demonstration of VDR in venule and capillary endothelial cells of normal human skin, in vivo, may indicate that the receptor functions to bind circulating, and/or locally synthesized 1,25(OH)₂D₃. Second, that BAEC contain 1 α -hydroxylase activity and also respond to the

addition of the hormone by altering their growth parameters, may indicate the existence of an autocrine loop of vitamin D metabolism. This autocrine loop would consist of the 1 α -hydroxylase generating the active hormone from its precursor 25(OH)D₃, and the receptor mediating the biological effects of the hormone. One such effect could be to downregulate both VDR and 1 α -hydroxylase by eliciting growth inhibition and terminal differentiation of the cells. This possibility of autocrine effects of vitamin D is of special interest in view of this observation that significant amounts of 1,25(OH)₂D₃ are generated at physiologic concentrations (10^{-8} M) of the 25(OH)D₃ precursor. In this context it is exciting to compare endothelial cells to T cells and macrophages, where activation elicits VDR expression (12) and 1 α -hydroxylase activity (40), respectively. Similarly, keratinocytes both synthesize and possess receptors for 1,25(OH)₂D₃ and these parameters vary according to the state of cell differentiation (6). Endothelial cells thus may represent yet another example of a vitamin D microendocrine system with potential relevance to cell differentiation as well as intercellular communication within a local environment.

The gene products activated by 1,25(OH)₂D₃ in endothelial cells remain to be defined, but one could hypothesize as to the potential role(s) of 1,25(OH)₂D₃ in endothelial cells. Endothelial cells form a selective barrier between the bloodstream and vessel wall. Under the influence of activating signals, endothelial cells express class II MHC antigens and other markers (41). It has been suggested that activated endothelial cells participate in antigen presentation and immune response. Because 1,25(OH)₂D₃ is now accepted as an immunoregulator with effects on both macrophages and T cells (12), the function of 1,25(OH)₂D₃ on endothelial cells could resemble its influence on the cellular immune system. Alternatively, nutritional studies have provided evidence that vitamin D or its metabolites accelerate atherogenesis. Although the mechanism of this effect remains unclear, this study shows that endothelial cells have the molecular machinery to react to 1,25(OH)₂D₃. Be-

cause injury to the endothelial lining is a key facet of the current theory of the pathogenesis of atherosclerosis, a negative or positive influence of $1,25(\text{OH})_2\text{D}_3$ in the reendothelialization process could be of great significance to determining the incidence of atherosclerotic plaque formation. Finally, abnormalities in the levels of calcium regulating hormones such as $1,25(\text{OH})_2\text{D}_3$ have been recognized in human and experimental hypertension (42, 43). Therefore, interactions of $1,25(\text{OH})_2\text{D}_3$ with endothelial cells as well as with vascular smooth muscle cells in the control of vascular tone (44–46) represent potentially exciting areas of investigation that could provide a link between the $1,25(\text{OH})_2\text{D}_3$ hormone and the pathophysiology of coronary artery disease.

Acknowledgments

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