

Selective expression of a normal action of the 1,25-dihydroxyvitamin D3 receptor in human skin fibroblasts with hereditary severe defects in multiple actions of that receptor.

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Research Article

We evaluated three actions of 1,25-dihydroxycholecalciferol [1,25-(OH)2D3] in human skin fibroblasts to test for heterogeneity in hormone-response coupling. In fibroblasts from normal subjects the 1,25-(OH)2D3 concentrations for half-maximal effect (EC50) were: for mitogenic effect 0.0001-0.0005 nM, for antimitogenic effect 1 nM, and for induction of 25-OHD3 24-hydroxylase (24-OHase) 5 nM. To evaluate the effects of mutations presumed to be in the gene for the 1,25-(OH)2D3 receptor we examined cell lines representing four kindreds with hereditary resistance to 1,25-(OH)2D3 ("mutant" cell lines). In one mutant cell line all three 1,25-(OH)2D3 actions were severely abnormal. In one mutant cell line 24-OHase induction and mitogenic action were undetectable, but EC50 and maximal effect were normal for antimitogenic action of 1,25-(OH)2D3. In two mutant cell lines 24-OHase induction and antimitogenic actions were undetectable or severely impaired but mitogenic action were undetectable or severely impaired but mitogenic action was normal in EC50 and normal or increased in maximal effect. The mitogenic and antimitogenic actions in normal cells showed a similar profile of potency ratios for 1,25-(OH)2D3 and six analogues. Whenever a mutant cell showed a normal or even an abnormal mitogenic or antimitogenic effect of 1,25-(OH)2D3, these effects showed potency ratios similar to wild type, suggesting mediation by a similar 1,25-(OH)2D3 receptor. We conclude that three 1,25-(OH)2D3 actions show important differences in hormone response coupling [...]

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Selective Expression of a Normal Action of the 1,25-Dihydroxyvitamin D₃ Receptor in Human Skin Fibroblasts with Hereditary Severe Defects in Multiple Actions of that Receptor

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Abstract

We evaluated three actions of 1,25-dihydroxycholecalciferol [1,25-(OH)₂D₃] in human skin fibroblasts to test for heterogeneity in hormone-response coupling. In fibroblasts from normal subjects the 1,25-(OH)₂D₃ concentrations for half-maximal effect (EC₅₀) were: for mitogenic effect 0.0001–0.0005 nM, for antimitogenic effect 1 nM, and for induction of 25-OHD₃ 24-hydroxylase (24-OHase) 5 nM. To evaluate the effects of mutations presumed to be in the gene for the 1,25-(OH)₂D₃ receptor we examined cell lines representing four kindreds with hereditary resistance to 1,25-(OH)₂D₃ (“mutant” cell lines). In one mutant cell line all three 1,25-(OH)₂D₃ actions were severely abnormal. In one mutant cell line 24-OHase induction and mitogenic action were undetectable, but EC₅₀ and maximal effect were normal for antimitogenic action of 1,25-(OH)₂D₃. In two mutant cell lines 24-OHase induction and antimitogenic actions were undetectable or severely impaired but mitogenic action was normal in EC₅₀ and normal or increased in maximal effect. The mitogenic and antimitogenic actions in normal cells showed a similar profile of potency ratios for 1,25-(OH)₂D₃ and six analogues. Whenever a mutant cell showed a normal or even an abnormal mitogenic or antimitogenic effect of 1,25-(OH)₂D₃, these effects showed potency ratios similar to wild type, suggesting mediation by a similar 1,25-(OH)₂D₃ receptor.

We conclude that three 1,25-(OH)₂D₃ actions show important differences in hormone response coupling indicated by differences in EC₅₀ for 1,25-(OH)₂D₃ and by different consequences of receptor mutations.

Introduction

It has been generally believed that the induction mechanism of all 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃)¹ receptor actions is the same, and that a defect in one 1,25-(OH)₂D₃ receptor action implies similar defects in all 1,25-(OH)₂D₃ receptor actions. However, certain actions of 1,25-(OH)₂D₃ in a

homogeneous cell population differ in 1,25-(OH)₂D₃ concentration for half-maximal effect (EC₅₀) (1), suggesting differences in the process of hormone-response coupling. Moreover, recent data suggest diverse mechanisms of action for other v-erb-A related receptor molecules (2–7). To characterize possible differences in 1,25-(OH)₂D₃ coupling to three presumably nuclear actions (mitogenic action, antimitogenic action, and induction of 25-OH-D₃-24-hydroxylase [24-OHase]) we studied 1,25-(OH)₂D₃ analogue specificity, EC₅₀, and effect of 1,25-(OH)₂D₃ receptor mutations.

Methods

Materials. Cell culture media were from Gibco Laboratories (Grand Island, NY); defined fetal bovine serum was from HyClone Laboratories (Logan, UT); 1 α ,25-(OH)₂D₃, 24,25-dihydroxycholecalciferol (24,25-(OH)₂D₃), 25-hydroxycholecalciferol (25-OH-D₃), 1 α ,25-(OH)₂-24,24F₂D₃ (RO-22-9343), and 1 α ,25-dihydroxy- Δ 16-23ynecholecalciferol (RO23-7553) were gifts from M. Uskokovic (Hoffmann-LaRoche, Nutley, NJ); 1 β ,25-dihydroxycholecalciferol (1 β ,25-(OH)₂D₃) was a gift from M. Holick and Rahul Ray (Boston University Medical School). Cholecalciferol and thymidine were from Sigma Chemical Co. (St. Louis, MO). Insulin (regular ileitin I, beef-pork) was from Eli Lilly & Co. (Indianapolis, IN). Hoechst dye 33342 was from Polysciences, Inc. (Warrington, PA). [*Methyl*-1',2'-³H]Thymidine (100–130 mCi/mmol sp act) and 1 α ,25-dihydroxy-[26,27-*methyl*-³H]cholecalciferol (sp act 176 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL). All other reagents were of the highest possible purity.

Calciferol analogues were always tested for purity on reverse-phase high performance liquid chromatography within 2 wk before use. Calciferols were dissolved in ethanol. Final concentration of ethanol in media for experiments was 0.1%, either with or without calciferol. 1,25-(OH)₂D₃ content of untreated serum was 39 pg/ml, and after charcoal treatment it was below the detection limit (5 pg/ml).

Cell culture. Two normal skin fibroblast lines were obtained from the American Type Culture Collection (Rockville, MD) and two additional lines were established from skin biopsies in our laboratory (8).

Three of four cell lines from patients with hereditary resistance to 1,25-(OH)₂D₃ (“mutant cell lines”)² had been tested previously to characterize their defects in 1,25-(OH)₂D₃ receptors (8–11) (Table I). The fourth cell line from a patient with hereditary resistance to 1,25-(OH)₂D₃ was kindly provided by Dr. K. Kruse (12), and we characterized it using the same methods (Table I).

Cells were grown in Dulbecco's minimal essential medium/10% fetal bovine serum/0.2 mM glutamine/80 mg per liter gentamycin/0.1 μ M insulin. Cells were maintained in 150-cm² plastic tissue culture flasks (Costar, Cambridge, MA), at 37°C in an atmosphere of 5% CO₂/95% air. Cells from passages 12–25 were used. All cell lines were tested for mycoplasma (American Type Culture Collection) with nega-

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1. Abbreviations used in this paper: 24,25-(OH)₂D₃, 24,25-dihydroxycholecalciferol; 25-OH-D₃, 25-hydroxycholecalciferol.

Table I. Characterization of Receptor Defects in Skin Fibroblasts from Subjects with Hereditary Resistance to 1,25-(OH)₂D₃

Cell line	1,25-(OH) ₂ D ₃ binding to receptor*	1,25-(OH) ₂ D ₃ receptor uptake by nuclei [‡]	1,25-(OH) ₂ D ₃ receptor binding to DNA [§]	Classification of 1,25-(OH) ₂ D ₃ receptor defect
10	Undetectable	NA	NA	Hormone-binding defect
11	Undetectable	Undetectable	NA	Hormone-binding defect
2	Normal	Undetectable	Normal	Cytosol-to-nucleus translocation defect
7	Normal	Mild decrease	Abnormal	DNA-binding defect

* Measured as [³H]1,25-(OH)₂D₃-binding at 4°C to extracts solubilized from cells with high KCl concentration (11). [‡] Measured as [³H]1,25-(OH)₂D₃ uptake into nuclei of intact cells at 37°C (11). [§] Measured as the elution position of [³H]1,25-(OH)₂D₃ in a KCl gradient applied to a DNA-cellulose column containing adsorbed receptors (previously bound to [³H]1,25-(OH)₂D₃) (8). NA, not analyzed.

tive results. Cells were incubated in serum-free medium for 24 h before experiments.

Induction of 24-OHase. All 24-OHase assays on normal or mutant cells were done as previously described (8), with minor modifications. These modifications include increase in cell number/tube (3 million/tube), and filtration of extracts prior to chromatography on a microfilter (Millipore HV 0.45 μm; Nichon Millipore, Kogyo, Japan) to prevent loss of materials on the guard column. The three mutant cell lines that had been tested previously (8, 10) were reanalyzed in one experiment. The fourth mutant cell line (12) and the normals were analyzed in two separate experiments in triplicate. All 1,25-(OH)₂D₃ was added in serum-free media, for 24 h.

Measurement of DNA synthesis by [³H]thymidine incorporation. Cells were subcultured to 24-vial plates (Costar). Experimental conditions were then optimized for testing either antimetabolic effect (A) or mitogenic effect (B) of calciferols. Each cell line was tested with both protocols at least four times.

For protocol A cells were used within 1 d after reaching confluency. They were placed into serum-free media for 24 h; then different concentrations of 1,25-(OH)₂D₃ or 1,25-(OH)₂D₃ analogue were added in serum-free medium for another 24 h.

For protocol B cells were used at half confluency and also maintained in serum-free medium for 24 h before testing; then 1,25-(OH)₂D₃ analogues were added in a medium supplemented with 0.2 μM insulin and with 10% calciferol-free bovine serum for another 24 h.

After 24 h the media were replaced with fresh aliquots of the same media (without or with calciferols) with [³H]thymidine at a final concentration of 1 μCi/ml, for another 24 h. The reaction was stopped with removal of medium, and cell monolayers were washed with ice-cold PBS containing 2 mM thymidine. Cell associated acid insoluble materials were precipitated by addition of 500 μl 5% trichloroacetic acid solution to the monolayers and incubation for 2 h at 4°C. Then the dried precipitate was solubilized with 1 N sodium chloride. Radioactivity was counted in a liquid scintillation counter, and data were expressed as percentage of counts in control wells without hormone added (mean ± 1 SE). Control values for different experiments were 5,700 ± 300 counts/min *n* = 504 for protocol A, and 3,000 ± 200 cpm *n* = 336 for protocol B. Normal cells and mutant cells always were included in the same assay, and their control values did not differ significantly.

Measurement of DNA content with microfluorimetry. Effects of 1,25-(OH)₂D₃ analogues on cell growth were monitored by serial measurement of DNA content with a rapid staining technique (13). For each experiment, cells were subcultured to six 96-well microtiter plates (USA Scientific Plastics, Ocala, FL) at 1,500 cells/well. Cells in microtiter plates were grown to confluency before testing (for protocol A), or to 75% confluency (for protocol B). Each cell line was tested under both conditions. Before calciferol exposure cells were washed and maintained in serum-free medium for 24 h. For antimetabolic effect (A) or mitogenic effect (B) cells were further cultured either in serum-free medium (A) or in medium supplemented with insulin and 10%

calciferol-free fetal bovine serum (B) without and with varying calciferol concentrations (between 10⁻¹⁴ and 10⁻⁶ M). Each plate included 16 replicates without calciferol added and 8 replicates at each calciferol concentration. Cells were incubated with test substances for 7 d, with media and test substances replaced every other day. After 24 h one plate was assayed for DNA content, and after 2, 3, 5, 6, 7 d further plates were taken for DNA measurement. During this 7-d period cell number in control wells (without hormone added) increased in protocol A to 145 ± 22% and in protocol B to 206 ± 64% of the cell number on day 0 (3,200 ± 1,800 *n* = 464 for protocol A, and 1,800 ± 700 *n* = 432 for protocol B).

DNA content was measured as follows: media were removed, then cells were washed with serum-free assay media (Eagle's No. 2. media with 50 mM tricine pH 7.4), and Hoechst 33342 stain solution (50 ng/ml in assay medium, 150 μl/well) was added. Cells were incubated with the staining solution for 60 min at 37°C. After removal of staining solution microfluorometric analysis was carried out using a MicroFLUOR reader (Dynatech Laboratories Inc., Chantilly, VA; excitation filter-365 nm, emission filters: 450 nm narrow band, UV blocking nonfluorescent filter 400 nm). Background was subtracted by measuring fluorescence in wells without cells in each experiment. In preliminary experiments a relationship between cell number in wells and fluorescence units was established. Using this linear relationship, we pooled data by converting fluorescence units to percent of cell number in control wells. In each experiment normal cells and cells with 1,25-(OH)₂D₃ receptor defects were studied together. We report response amplitude on the day of maximal amplitude. There were no significant variations in half maximally effective hormone dose between days 1 and 7.

Statistics. Data were evaluated by analysis of variance, Student *t* test, and Scheffé's test for comparison of multiple means. Data are expressed as mean ± 1 SD, except where indicated. EC₅₀s were estimated by visual interpolation from curves of pooled data.

Results

Effects of 1,25-(OH)₂D₃ on normal human skin fibroblasts
1,25-(OH)₂D₃ effect on 24-OHase activity. 1,25-(OH)₂D₃ stimulated 24-OHase with a maximum stimulation (25–40-fold above baseline) at 10⁻⁷ M 1,25-(OH)₂D₃. Half maximal effect occurred at 5 nM 1,25-(OH)₂D₃ (Fig. 1).

Antimetabolic effect of 1,25-(OH)₂D₃. 1,25-(OH)₂D₃ inhibited [³H]thymidine incorporation in normal skin fibroblasts. Under serum-free condition (Fig. 2, left) the maximal inhibitory effect reached 18 ± 4% of control, with an EC₅₀ at 1 nM 1,25-(OH)₂D₃. In the presence of serum (Fig. 2, right) the maximal inhibitory effect was similar (21 ± 3), but the EC₅₀ was at 10-fold higher 1,25-(OH)₂D₃ concentration (10 nM).

Using microfluorimetry we also found an inhibitory effect of 1,25-(OH)₂D₃ on cell growth (Fig. 3, left). This effect of

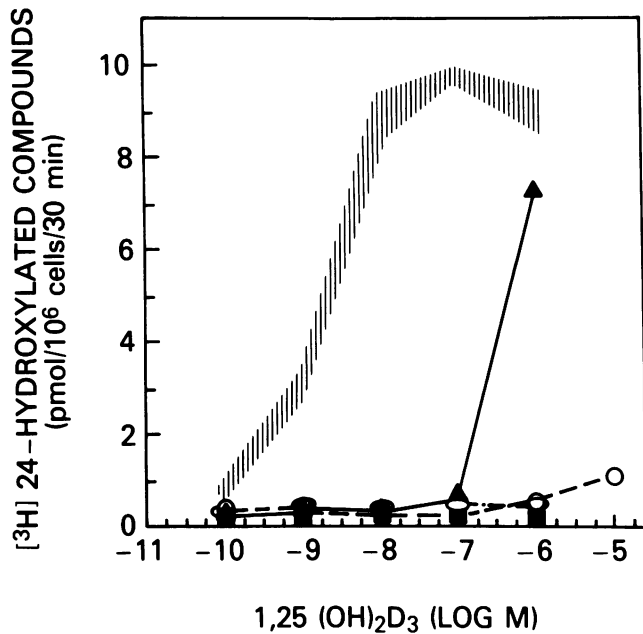


Figure 1. Induction of 25-OHD₃-24-hydroxylase activity by 1,25-(OH)₂D₃ in normal and in mutant skin fibroblasts (normal: striped zone; line 11: ○; line 7: ■; line 2: ▲; line 10: ○). Means±SD were pooled from previous data (8, 10) and from additional recent data. Standard errors are equal to or less than 8% of mean.

1,25-(OH)₂D₃ was greater ($P < 0.01$) without ($39 \pm 10\%$) than with serum ($65 \pm 7\%$) (at 10^{-7} M 1,25-(OH)₂D₃ at the sixth day of a 7-d incubation). Half maximal inhibition on days 1–7 was found at 1 nM 1,25-(OH)₂D₃ in serum-free, and 30 nM 1,25-(OH)₂D₃ in serum supplemented media.

Mitogenic effect of 1,25-(OH)₂D₃. Stimulatory effect of 1,25-(OH)₂D₃ on [³H]thymidine incorporation in normal fibroblasts was reproducible only after we optimized the incubation conditions. Without serum, with serum from another source, or without insulin this effect was not detectable in normal fibroblasts (for example, see Fig. 2, left). Under our optimized conditions (Fig. 2, right) [³H]thymidine incorporation was stimulated by 1,25-(OH)₂D₃ maximally ($148 \pm 3\%$ of control) at 0.01 nM 1,25-(OH)₂D₃. EC₅₀ for mitogenic action was 0.0005 nM 1,25-(OH)₂D₃.

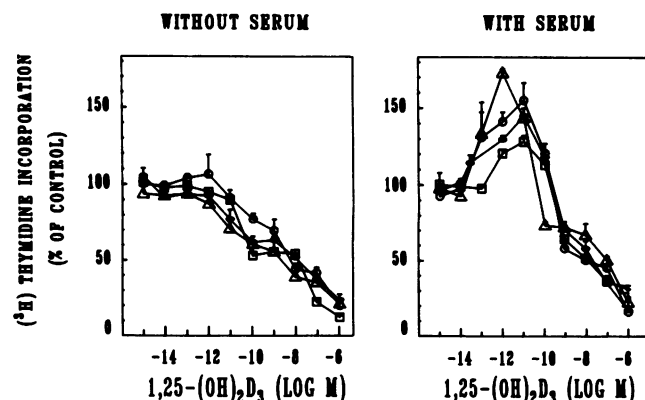


Figure 2. Effect of 1,25-(OH)₂D₃ on DNA synthesis in normal skin fibroblasts. Data are mean±1 SE from 22 experiments in triplicate, on four different cell lines. (Left) Without serum. (Right) With serum.

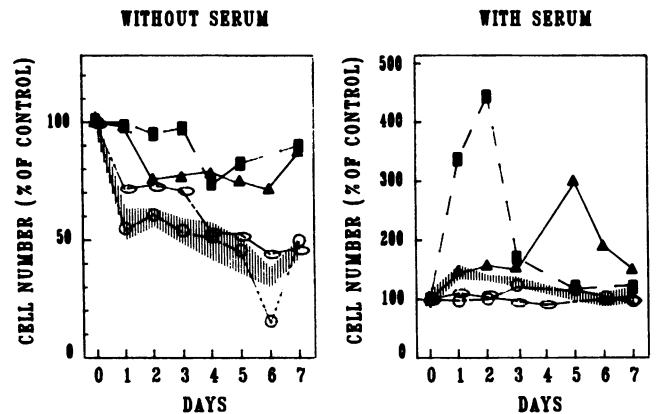


Figure 3. Effect of 1,25-(OH)₂D₃ on total DNA synthesis, measured by microfluorimetry, in normal human skin fibroblasts (striped area), and in fibroblasts from patients with hereditary resistance to 1,25-(OH)₂D₃ (line 11, ○; line 7, ■; line 2, ▲; line 10, ○). Data (mean±1 SE) represent percent of cell number at each day compared to cell number without hormone on the same day of incubation. Each line was tested in at least three experiments. Error ranges are given only for normal cells; in all other lines errors are equal to or less than 9% of the mean. (Left) Maximal antimitogenic effect of 1,25-(OH)₂D₃ (10^{-7} M for normal cells and for mutant cell line subject 11, and 10^{-6} M for mutant cell lines 10, 2, and 7). (Right) Maximal mitogenic effect of 1,25-(OH)₂D₃ (at 10^{-11} M for normal fibroblasts and for mutant cell lines 7, and 2, and at 10^{-10} M for mutant cell lines 10 and 11).

Using microfluorometric measurement of DNA content, we were also unable to detect a mitogenic action of 1,25-(OH)₂D₃ in the absence of serum and insulin. In the presence of serum and insulin, however, mitogenic action occurred after 24 h incubation with hormone (Fig. 3, right). This action decreased with longer 1,25-(OH)₂D₃ exposure, and became undetectable after 5 d. Maximal stimulation ($146 \pm 23\%$) occurred after 24 h at 0.005 nM 1,25-(OH)₂D₃ with an EC₅₀ of 0.0001 nM 1,25-(OH)₂D₃.

1,25-(OH)₂D₃ actions in cell line 10 from subject with hereditary resistance to 1,25-(OH)₂D₃

This mutant cell line was classified as having defective hormone-binding to receptors (Table I).

1,25-(OH)₂D₃ effect on 24-OHase activity. Stimulatory effect of 1,25-(OH)₂D₃ on 24-OHase activity in cell line 10 was highly abnormal (Fig. 1). There was no significant increase between 10^{-11} and 10^{-7} M 1,25-(OH)₂D₃; 10^{-6} M 1,25-(OH)₂D₃ caused only 2.3-fold and 10^{-5} M 1,25-(OH)₂D₃ a 4.6-fold increase in 24-OHase activity. In the same experiments 10^{-7} M 1,25-(OH)₂D₃ caused a 40-fold increase in normal cells.

Antimitogenic effect of 1,25-(OH)₂D₃. Under serum-free conditions (Fig. 4, left) the maximal inhibitory effect ($16 \pm 4\%$) of 10^{-6} M 1,25-(OH)₂D₃ was within the normal range, but EC₅₀ (10 nM) was 10-fold greater than normal. In the presence of serum (Fig. 4, right) the maximal inhibitory effect ($63 \pm 4\%$ of baseline) was less ($P < 0.001$) than in normal cells ($21 \pm 4\%$), and the EC₅₀ for 1,25-(OH)₂D₃ (100 nM) was 100-fold greater than normal.

Using microfluorometric detection of DNA content, maximal inhibitory action of 1,25-(OH)₂D₃ with serum-free media

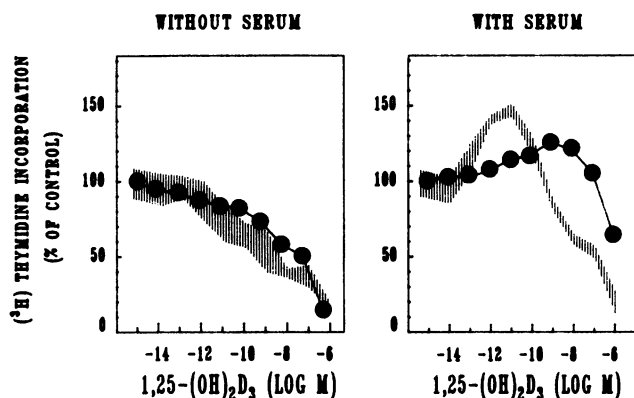


Figure 4. Effect of 1,25-(OH)₂D₃ on DNA synthesis in a skin fibroblast line derived from patient 10 with hereditary resistance to 1,25-(OH)₂D₃. Data are mean ± 1 SE of 10 experiments (left) or of 5 experiments (right). Symbols accommodate errors. Normal range is given as a striped zone, based on pooled data from Fig. 2.

was within the normal range, reaching maximum on the 6th day (17 ± 8% of control), and EC₅₀ was increased 10-fold to 10 nM. In the presence of serum the inhibitory action was less (maximum inhibition 68% ± 5% of control on the 7th day) than in normal cells (*P* < 0.05), and EC₅₀ was 100 nM.

Mitogenic effect of 1,25-(OH)₂D₃. The stimulatory effect of 1,25-(OH)₂D₃ on [³H]thymidine incorporation was detectable in serum-supplemented media (maximum 128 ± 6%, and EC₅₀ 100-fold greater than normal) (Fig. 4, right). The maximal increase in DNA content (123 ± 2% of control by microfluorimetry) occurred after 3 d of hormone exposure, with an EC₅₀ of 0.1 nM.

1,25-(OH)₂D₃ actions in cell line 11 from subject with hereditary resistance to 1,25-(OH)₂D₃

This mutant cell line was classified as having calcitriol receptors with hormone-binding defect (Table I).

1,25-(OH)₂D₃ effect on 24-OHase activity. 24-OHase in cell line 11 was not induced by any 1,25-(OH)₂D₃ dose up to 10⁻⁵ M (Fig. 1).

Antimitogenic effect of 1,25-(OH)₂D₃. 1,25-(OH)₂D₃ inhibited [³H]thymidine incorporation in cell line 11 (8 experiments) (Table II) exactly as in normal cell lines. Under serum-free conditions (Fig. 5, left) maximal inhibitory effect reached 14 ± 3% of control with an EC₅₀ at 0.5 nM 1,25-(OH)₂D₃. In the presence of serum (Fig. 5, right) the maximal inhibitory effect was 45 ± 4% of control, and the EC₅₀ for 1,25-(OH)₂D₃ was the same as in the normal cells in four experiments.

Cell line 11 (tested with or without serum) also showed normal inhibitory action of 1,25-(OH)₂D₃ on cell growth measured by microfluorimetry. Maximum inhibition was reached on day six (55 ± 5% with and 45 ± 3% without serum at 10⁻⁷ M 1,25-(OH)₂D₃). The concentration for half maximal inhibition was also normal, 0.5 nM 1,25-(OH)₂D₃ in serum-free media and 10 nM in serum-supplemented media.

Mitogenic effect of 1,25-(OH)₂D₃. The stimulatory effect of 1,25-(OH)₂D₃ on [³H]thymidine incorporation was not detectable in four experiments (Fig. 5, right). We were unable to find stimulatory effect also with microfluorimetry.

1,25-(OH)₂D₃ actions in cell line 7 from subject with hereditary resistance to 1,25-(OH)₂D₃

This mutant cell line was classified as showing 1,25-(OH)₂D₃ receptors with a DNA-binding defect (Table I).

1,25-(OH)₂D₃ effect on 24-OHase activity. There was no 24-OHase induction detected even with the highest hormone concentrations (7) (Fig. 1).

Antimitogenic effect of 1,25-(OH)₂D₃. In these cells 1,25-(OH)₂D₃ inhibited [³H]thymidine incorporation only at very high dose in the presence or absence of serum. With serum, maximal inhibition was 35 ± 11% of control (Fig. 6, right). Without serum, inhibition of [³H]thymidine incorporation was 50 ± 2% of control. EC₅₀ was 1,000-fold greater than normal (Fig. 6, left).

DNA quantitation by microfluorimetry revealed a similar defect in this 1,25-(OH)₂D₃ action. There was only a slight inhibitory action (74 ± 2% of control) after 4 d with 10⁻⁶ M 1,25-(OH)₂D₃.

Mitogenic effect of 1,25-(OH)₂D₃. Mutant cell line 7 retained a normal or even exaggerated stimulatory action of 1,25-(OH)₂D₃ on DNA synthesis. Stimulatory effect on [³H]thymidine incorporation in the presence of serum at 10⁻¹³ and 10⁻¹² M 1,25-(OH)₂D₃ was the same as in normal cells (Fig. 6, right). The maximal stimulatory effect (245 ± 9%) at 10⁻¹¹ M 1,25-(OH)₂D₃ was greater than the normal maximal stimulatory effect (*P* < 0.001) in nine experiments.

Microfluorometric measurement of DNA content revealed similarly accentuated stimulatory effect reaching a maximum (444 ± 6%) after 48 h hormone exposure at 10⁻¹² M 1,25-(OH)₂D₃, in the presence of serum (Fig. 3).

1,25-(OH)₂D₃ effects in cell line 2 from subject with hereditary resistance to 1,25-(OH)₂D₃

This mutant cell line was classified as having 1,25-(OH)₂D₃ receptors with cytosol-to-nucleus translocation defect (Table I).

Table II. Summary of Three 1,25-(OH)₂D₃ Actions in Four Cell Lines from Subjects with Hereditary Resistance to 1,25-(OH)₂D₃

Cell line	Classification of 1,25-(OH) ₂ D ₃ receptor defect	1,25-(OH) ₂ D ₃ action		
		24OHase induction	Mitogenic action	Antimitogenic action
10	Hormone-binding defect	Abnormal*	Abnormal	Abnormal
11	Hormone-binding defect	Undetectable	Undetectable	Normal
7	DNA-binding defect	Undetectable	Normal or increased	Undetectable
2	Cytosol-to-nucleus translocation defect	Abnormal	Normal or increased	Abnormal

* Abnormal implies detectable action (with normal or deficient amplitude) but abnormally increased EC₅₀ for 1,25-(OH)₂D₃.

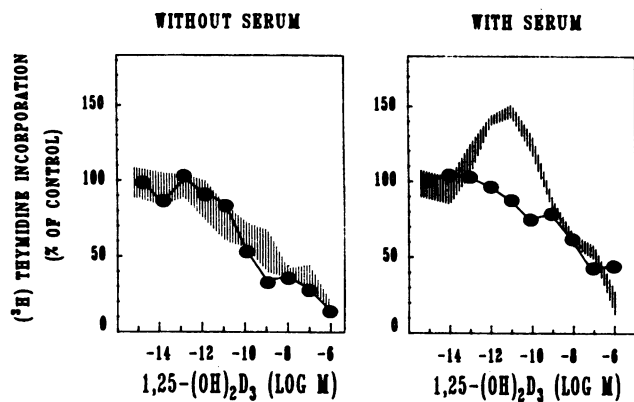


Figure 5. Effect of 1,25-(OH)₂D₃ on DNA synthesis in a skin fibroblast line from patient 11 with hereditary resistance to 1,25-(OH)₂D₃. Data are mean ± 1 SE of eight (left) or four (right) experiments. Symbols accommodate errors. Normal range is given as a striped zone based on pooled data from Fig. 2.

1,25-(OH)₂D₃ effect on 24-OHase activity. There was no increase in 24-OHase activity up to 10⁻⁷ M 1,25-(OH)₂D₃, but at very high 1,25-(OH)₂D₃ concentrations 24-OHase was induced to a normal maximal activity (7) (Fig. 1).

Antimitogenic effect of 1,25-(OH)₂D₃. In cell line 2 inhibitory effect of 1,25-(OH)₂D₃ on DNA synthesis was highly abnormal. In the presence of serum (Fig. 7, right) we could not detect inhibition of [³H]thymidine incorporation at any 1,25-(OH)₂D₃ dose. In the absence of serum (Fig. 7, left) inhibitory action of 1,25-(OH)₂D₃ on [³H]thymidine incorporation was attenuated: maximal inhibitory effect was 45 ± 8% of control, the EC₅₀ for 1,25-(OH)₂D₃ was increased 1,000-fold above normal.

Using microfluorimetry we found inhibitory effect on cell growth only with 10⁻⁶ M 1,25-(OH)₂D₃ under serum-free conditions.

Mitogenic effect of 1,25-(OH)₂D₃. In cell line 2 the mitogenic effect of 1,25-(OH)₂D₃ was retained in approximately normal fashion. Stimulatory effect of 1,25-(OH)₂D₃ on [³H]thymidine incorporation started with the same hormone concentrations as in normal cells, but it increased to a greater

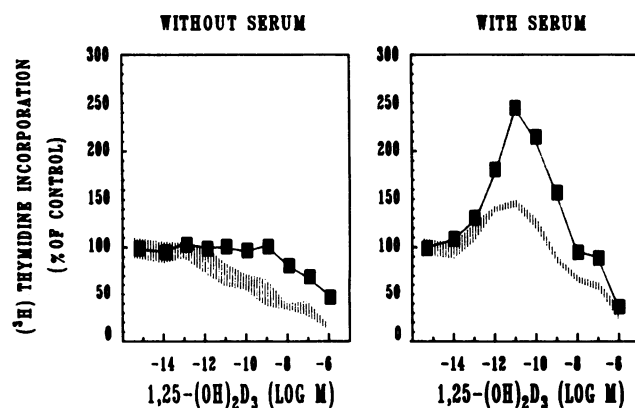


Figure 6. Effect of 1,25-(OH)₂D₃ on DNA synthesis in a skin fibroblast line derived from patient 7 with hereditary resistance to 1,25-(OH)₂D₃. Data are mean ± 1 SE of five experiments (left) or of nine experiments (right). Symbols accommodate errors. Normal range is given as striped zone, based on pooled data from Fig. 2.

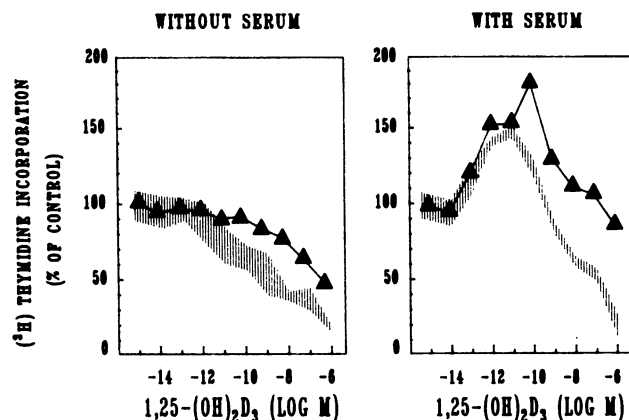


Figure 7. Effect of 1,25-(OH)₂D₃ on DNA synthesis in a skin fibroblast line derived from patient 2 with hereditary resistance to 1,25-(OH)₂D₃. Data are mean ± 1 SE of six experiments (left) or of eight experiments (right). Symbols accommodate errors. Normal range is given as striped zone based on pooled data from Fig. 2.

maximum (185 ± 7%; *P* < 0.01 compared to normal maximum) at 10⁻¹⁰ M 1,25-(OH)₂D₃ (Fig. 7, right).

Using microfluorimetry we found a time-dependent increase in mitogenic effect with a maximum of 300% increase on the 5th day at 10⁻¹² M 1,25-(OH)₂D₃, in the presence of serum (Fig. 3).

1,25-(OH)₂D₃ analogue specificity for mitogenic or antimitogenic actions in normal cells

The selective retention of normal 1,25-(OH)₂D₃ stimulatory action on DNA synthesis in some mutant cells (lines 2 and 7), and of normal 1,25-(OH)₂D₃ inhibitory action in another (line 11) with severe defect in 1,25-(OH)₂D₃ receptor actions raised the question whether these effects in normal cell lines or in mutant cell lines are 1,25-(OH)₂D₃ receptor mediated. To address this we studied 1,25-(OH)₂D₃ analogue specificity of these actions in normal cells and in mutant cells.

1,25-(OH)₂D₃ and six analogues showed in normal cells indistinguishable order of potency for mitogenic and antimitogenic actions (though the EC₅₀s for the two actions differed strikingly) (Table III). The relative unresponsiveness of normal cells to both actions of 1,25-(OH)₂D₃ established stereospecificity for these actions at the 1-hydroxyl residue.

1,25-(OH)₂D₃ analogue specificity for mitogenic or antimitogenic actions in mutant cells

In similar experiments, each response to 1,25-(OH)₂D₃ that was expressed in a normal manner by a mutant cell line showed analogue potency ratios indistinguishable from normal (compare the ratios for mutant cell line 11 in Table IV or mutant cell lines 7 and 2 in Table V to normal in Table III).

Responses that were defective (in 1,25-(OH)₂D₃ maximal effect or EC₅₀) but measurable in mutant cells also showed normal analogue potency ratios (mutant cell lines 2 and 10 in Table IV or line 10 in Table V compared to normal in Table III).

Discussion

Our results indicate important differences in induction mechanisms for three 1,25-(OH)₂D₃ actions. We have now found

Table III. EC₅₀s of 1,25-(OH)₂D₃ and Its Analogues for Mitogenic and Antimitogenic Action in Normal Skin Fibroblasts*

Vitamin D ₃ analogue	EC ₅₀ for mitogenic action		EC ₅₀ for antimitogenic action	
	nM	Ratio [‡]	nM	Ratio [‡]
1α,25-(OH) ₂ -Δ16-23yne-D ₃	0.0001	5	0.1	10
1α,25-(OH) ₂ -24,24F ₂ D ₃	0.0005	1	0.5	2
1α,25-(OH) ₂ D ₃	0.0005	1	1	1
24,25-(OH) ₂ D ₃	0.001	0.5	10	0.1
25-OHD ₃	0.1	0.005	200	0.005
1β,25-(OH) ₂ D ₃	0.5	0.001	100	0.01
D ₃	50	0.00001	500	0.002

* All data are means from at least two experiments in triplicate. These EC₅₀s were derived from cell proliferation assays, based on [³H]thymidine incorporation. Mitogenic assays used a serum and insulin supplemented medium, and antimitogenic assays used a medium without serum or insulin. Calciferol analogues were present for 48 h, with [³H]thymidine added for the last 24 h.

[‡] Ratio between EC₅₀ for 1α,25-(OH)₂D₃ and EC₅₀ for a vitamin D₃ analogue.

that some mutant cell lines can selectively retain at least one 1,25-(OH)₂D action (mitogenic or antimitogenic action) in an approximately normal fashion while 24-OHase and some other actions are severely abnormal.

We have established incubation conditions in human skin fibroblasts to optimize mitogenic and antimitogenic effects of 1,25-(OH)₂D₃. Our observations with different media suggest that the mitogenic effect of 1,25-(OH)₂D₃ depends upon the presence of serum (i.e., presumably growth factors in serum) and insulin (presumably IGF-1/somatomedin-C since insulin acts on the latter's receptor at the high concentrations we employed [14]). Prior reports have also suggested that the mitogenic action of 1,25-(OH)₂D₃ may involve serum or growth factors (15), such as PDGF (15), interleukins (16), or epidermal growth factor (16). We also found that confluency status had to be optimized, supporting prior studies on the importance of confluency (17) or differentiation (18).

We observed biphasic effects of 1,25-(OH)₂D₃ on growth of normal skin fibroblasts, using a short-term assay based on [³H]thymidine incorporation or using a long-term assay based on total DNA content. Biphasic 1,25-(OH)₂D₃ effects with stimulation of DNA synthesis at low doses and inhibition at higher doses have been observed previously in normal or malignant cells (19–21).

Antimitogenic effects of 1,25-(OH)₂D₃ on DNA synthesis have been observed in many cell systems, often accompanied by induction of cell differentiation (20, 22).

Mitogenic effects of 1,25-(OH)₂D₃ on DNA synthesis have been described in embryonic fibroblasts (22), in cartilage (17), in T lymphocytes (23), and in monocytes (24). While mitogenic effects of 1,25-(OH)₂D₃ have been found in only few systems and have been of modest magnitude, the strikingly lower EC₅₀ for mitogenic than for antimitogenic effect in our studies and in prior studies (19–22) suggests that the mitogenic effect is physiologically important.

In mutant cell lines 2 and 7 with the most severe deficiency of antimitogenic effect, we noted an exaggerated amplitude of the mitogenic effect. Apparently the biphasic 1,25-(OH)₂D₃ dose-response relations result from interactions of two opposing effects; loss of one 1,25-(OH)₂D₃ effect (by selective assay conditions or by receptor mutation) allows the opposing one to be seen more clearly. The exaggerated mitogenic effects of 1,25-(OH)₂D₃ in cell lines 2 and 7 showed time courses different from normal and from each other (Fig. 3, right); the determinants of these time courses are not known.

We found EC₅₀ differences by as much as 10,000-fold among three 1,25-(OH)₂D₃ actions in normal cells. EC₅₀s for 1,25-(OH)₂D₃ actions were: 0.0001–0.0005 nM for mitogenic effect, 1 nM for antimitogenic effect (without serum or insulin), and 5 nM for 24-OHase induction. Differences in EC₅₀ among several 1,25-(OH)₂D₃ actions were also shown previously (1). Heterogeneity in EC₅₀ among multiple actions of other steroids have also been documented (25), but the molecular basis for this form of heterogeneity in action of 1,25-(OH)₂D₃ or other steroids has not been determined.

The absolute value of an EC₅₀, per se, must be interpreted with caution; we and others (24) noted a 10-fold difference in the EC₅₀ for antimitogenic action of 1,25-(OH)₂D₃ depending on serum addition, and EC₅₀ for mitogenic actions were gener-

Table IV. EC₅₀s for Antimitogenic Effect of 1,25-(OH)₂D₃ and Its Analogues in Cell Lines 11, 10, and 2 from Subjects with Hereditary Resistance to 1,25-(OH)₂D₃*

Vitamin D ₃ analogue	EC ₅₀ for antimitogenic action by cell line					
	Cell line 11		Cell line 10		Cell line 2	
	nM	Ratio [‡]	nM	Ratio	nM	Ratio
1α,25-(OH) ₂ -Δ16-23yne-D ₃	0.5	1	1	10	50	10
1α,25-(OH) ₂ -24,24F ₂ D ₃	NA		NA		500	1
1α,25-(OH) ₂ D ₃	0.5	1	10	1	500	1
24,25-(OH) ₂ D ₃	1	0.5	20	0.5	1,000	0.5
25-OHD ₃	50	0.01	100	0.1	1,000	0.5
1β,25-(OH) ₂ D ₃	100	0.005	500	0.02		No effect
D ₃	500	0.001	1,000	0.01		No effect

* All data are means from at least three experiments. These EC₅₀s were derived from cell proliferation assays, based on [³H]thymidine incorporation. Antimitogenic assays used a medium without serum, with calciferol analogue for 48 h, and with [³H]thymidine for the last 24 h.

[‡] Ratio between EC₅₀ for 1α,25-(OH)₂D₃ in that cell line and EC₅₀ for a vitamin D₃ analogue. NA, not analyzed.

Table V. EC₅₀s for Mitogenic Actions of 1,25-(OH)₂D₃ and Its Analogues in Cell Lines 7, 10, and 2 from Subjects with Hereditary Resistance to 1,25-(OH)₂D₃*

Vitamin D ₃ analogue	EC ₅₀ for mitogenic action by cell line					
	Cell line 7		Cell line 10		Cell line 2	
	nM	Ratio [†]	nM	Ratio	nM	Ratio
1 α ,25-(OH) ₂ - Δ 16-23yne-D ₃	NA		0.1	1	0.0001	10
1 α ,25-(OH) ₂ -24,24F ₂ D ₃	0.0002	8	0.1	1	0.001	1
1 α ,25-(OH) ₂ D ₃	0.001	1	0.1	1	0.001	1
24,25-(OH) ₂ D ₃	0.001	1	1	0.1	0.001	1
25-OHD ₃	0.07	0.14	10	0.01	10	0.0001
1 β ,25-(OH) ₂ D ₃	1	0.001	5	0.02	100	0.00001
D ₃	20	0.00005	20	0.005	50	0.00002

* All data are means from at least three experiments. These EC₅₀s were derived from cell proliferation assays, based on [³H]thymidine incorporation. Mitogenic assays used a serum and insulin supplemented medium with calciferol analogues for 48 h, and with [³H]thymidine for the last 24 h. NA, not analyzed. [†] Ratio between EC₅₀ for 1 α ,25-(OH)₂D₃ in that cell line and EC₅₀ for a vitamin D₃ analogue.

ally lower for long-term cell growth (microfluorimetry) than for the short-term [³H]thymidine incorporation) assay. The EC₅₀ for antimitogenic effect of 1,25-(OH)₂D₃ varied more than 10-fold among a spectrum of myeloid cell lines grown under identical conditions (18). These limitations do not apply to studies, such as ours or others (1), in which markedly differing EC₅₀s are manifested as biphasic curves in cells tested under a single condition.

Our studies of fibroblasts with hereditary defects in the 1,25-(OH)₂D₃ receptor revealed evidence for heterogeneity in mechanisms of coupling between hormone and response; certain mutations impaired several 1,25-(OH)₂D₃ actions but allowed another action to remain normal. Among the four lines we tested from subjects with hereditary resistance to 1,25-(OH)₂D₃ one line showed selective retention of a normal antimitogenic effect of 1,25-(OH)₂D₃, two showed selective retention of a normal mitogenic effect of 1,25-(OH)₂D₃, and one showed abnormalities in all 1,25-(OH)₂D₃ effects. Since the molecular sites of mutations (most or all mutations are presumed to be in the 1,25-(OH)₂D₃ receptor gene) in these cells are not yet known, and since we were not able to study larger numbers of mutant cell lines, we cannot directly relate the retained 1,25-(OH)₂D₃ actions to any characteristic alterations of receptor structure. In fact, in other skin fibroblast lines or peripheral mononuclear cells from subjects with hereditary resistance to 1,25-(OH)₂D₃ and undetectable hormone-binding to receptors, there was no retention of antimitogenic effect of 1,25-(OH)₂D₃ (26, 27). On the other hand, peripheral mononuclear cells showing a DNA-binding defect seemed to retain a subtle mitogenic effect of 1,25-(OH)₂D₃ (that was not commented upon) (27). Retention of the normal inhibitory effect of 1,25-(OH)₂D₃ on *c-myc* mRNA synthesis in a 1,25-(OH)₂D₃ resistant HL-60 cell line was also reported recently (28).

The selective retention of normal mitogenic or antimitogenic responses to 1,25-(OH)₂D₃ in some lines from subjects with hereditary resistance to 1,25-(OH)₂D₃ raised the question whether all these actions are mediated by the same receptor. To address this question we tested 1,25-(OH)₂D₃ analogue specificity of these actions in normal or in mutant cell lines. In normal cells the mitogenic and antimitogenic actions showed

similar relative potencies among 1,25-(OH)₂D₃ and six analogues, suggestive of mediation by the same receptor. Others also reported similar potency ratios for mitogenic and antimitogenic actions of 1,25-(OH)₂D₃ and several analogues (19, 21, 22, 29). Retained responses in mutant cell lines also showed analogue potency ratios similar to normal, suggesting mediation by a 1,25-(OH)₂D₃ receptor. Our data indicate that three actions of 1,25-(OH)₂D₃, differing by EC₅₀ and affected differently by receptor mutations, are mediated by receptors with shared hormone-binding characteristics.

Our data suggest that a mutation compromising several actions of the 1,25-(OH)₂D₃ receptor may leave other 1,25-(OH)₂D₃ receptor functions intact. These phenomena might have clinical importance. One of the patients (subject 11), whose cells displayed abnormal antimitogenic effect, responded to high doses of vitamin D₃ with suppression of PTH secretion but without an intestinal response³ (12). Since the 1,25-(OH)₂D₃ receptor action in the parathyroid might involve suppression of gene expression (30) while that in the intestine may involve stimulation of gene expression, differences in coupling mechanisms for these two effects are possible. Prior studies showed that 1,25-(OH)₂D₃ induction of 24-hydroxylase correlated well with calcemic response to 1,25-(OH)₂D₃.³ Our current findings show that mitogenic or antimitogenic response to 1,25-(OH)₂D₃ predict calcemic responsivity less well.

Studies of the v-erbA related family of receptors have suggested several potential mechanisms for heterogeneity of receptor coupling to responses. Heterogeneity could reflect differing effects from multiple forms of hormone receptor. These receptor subspecies can be encoded by one gene (31) or by several genes (32). To date there is no evidence for multiple genes or for alternate transcripts from one gene for the 1,25-(OH)₂D₃ receptor. Alternately, one receptor could couple differently to distinct responses; in particular, various functional domains of the same receptor can mediate coupling to

3. The calcemic response to calciferols, which was actually measured, undoubtedly involves multiple components, but the principal 1,25-(OH)₂D₃ action in raising serum calcium is at the level of calcium transport across the intestinal mucosa.

different target genes (5). The amino terminus of the progesterone receptor has been shown to help select the target gene, and a synthetic receptor construct was shown to retain selectively one but not another effect (33). Some steroid receptor target genes have variable numbers of receptor recognition elements, thereby presenting opportunities for varying cooperative effects in receptor actions (2, 4, 6). Heterogeneous coupling mechanisms are also possible through receptor interactions with an increasing spectrum of transcription factors (34, 35). For example, in certain target genes, no receptor recognition element may be present, and the receptor may modulate the gene by interacting with another DNA-binding protein (5, 36). In addition to transcriptional actions, posttranscriptional effects have been suggested for 1,25-(OH)₂D₃ (37), and other steroids (7, 38).

While we do not know if our molecular classification of receptor defects will predict similar patterns of response retention in other mutant cell lines, we present below several speculations to illustrate how the observed response patterns might arise.

Cell line 11 with a defect in 1,25-(OH)₂D₃-binding to receptor retained a normal antimitogenic effect of 1,25-(OH)₂D₃. Since the antimitogenic effect showed the analogue specificity characteristic of a 1,25-(OH)₂D₃ receptor, we postulate mediation by receptors present in such small numbers or with such low 1,25-(OH)₂D₃ affinity that they are beneath the detection limit of the assay of [³H]-1,25-(OH)₂D₃-binding to soluble extract. This could be analogous to a glucocorticoid resistant lymphoma cell line, which retained one glucocorticoid receptor function normally in the face of reduced numbers of glucocorticoid receptors (3). Such a postulated receptor subpopulation, arising within a presumably homogeneous cell line, must also have features that target it selectively to one or a limited number among target elements.

Cell line 7 with a defect in receptor-binding to DNA retained a normal (or even exaggerated) mitogenic effect of 1,25-(OH)₂D₃. We speculate that the 1,25-(OH)₂D₃ receptor may interact indirectly with its target for this effect by interacting directly with another protein. Such a 1,25-(OH)₂D₃ action need not require an intact DNA-binding domain of the 1,25-(OH)₂D₃ receptor (5, 36). In fact, the retained mitogenic effect in mutant lines 2 and 7 with defective nuclear interactions of receptor suggests this effect could be at a cytoplasmic target of the receptor.

We have shown that 1,25-(OH)₂D₃ coupling to three different actions differs strikingly in EC₅₀ and in dependency on receptor functions that are compromised by certain mutations. This heterogeneity implies that full information on the normal mechanism of 1,25-(OH)₂D₃ receptor actions will require detailed studies of multiple responses. 1,25-(OH)₂D₃ effects on DNA synthesis seem to provide indices for two distinct receptor functions, while induction of 24-hydroxylase activity measures a third that correlates best of these three with the calcemic effect³ of 1,25-(OH)₂D₃.

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