Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 1223-1226, February 1988
Medical Sciences

Receptor-mediated rapid action of 1α,25-dihydroxycholecalciferol: Increase of intracellular cGMP in human skin fibroblasts

(steroid/signal transduction/nongenomic/calcium)

JULIA BARSONY AND STEPHEN J. MARX

Mineral Metabolism Section, National Institute of Diabetes, Digestive, and Kidney Diseases, Bethesda, MD 20892

Communicated by DeWitt Stetten, Jr., October 12, 1987

ABSTRACT **The intracellular cGMP concentration in normal human cultured fibroblasts was increased 2- to 3-fold by 1α,25-dihydroxycholecalciferol [1α,25-$(OH)_2D_3$] in a dose-dependent manner between 0.01 nM and 1 μM. The response was detectable within 1 min, reached a maximum (225% ± 8% of baseline) at 6–8 min, and was no longer detectable at 30 min. The half-maximal effect of 1α,25-$(OH)_2D_3$ was at 1.8 nM, and 24,25-dihydroxycholecalciferol showed an estimated EC_{50} 100-fold higher. 1β,25-Dihydroxycholecalciferol, 25-hydroxycholecalciferol, and cholecalciferol had no detectable effect. Human skin fibroblasts with three different types of 1α,25-$(OH)_2D_3$ receptor defect did not respond to 1α,25-$(OH)_2D_3$ exposure with cGMP increase; however, the same cells (like normal cells) responded to testosterone or sodium nitroprusside with a rapid rise of cGMP. We conclude that the rapid rise of cGMP in response to calciferols shows an EC_{50} for 1α,25-$(OH)_2D_3$, a cholecalciferol analog specificity, and a cell line dependency that are all suggestive of mediation through a specific 1α,25-$(OH)_2D_3$ receptor.**

Over the past 15 years, studies from a number of laboratories have provided evidence that 1α,25-dihydroxycholecalciferol [1α,25-$(OH)_2D_3$ or calcitriol] acts like a steroid hormone through receptor-mediated regulation of nuclear events. However, rapid, presumably nongenomic, actions of 1α,25-$(OH)_2D_3$ (similar to those of other steroid hormones) have been documented in several systems (1). The most widely accepted explanation is that the rapid actions of steroids are receptor independent; direct action on the cell membrane modulates its lipid composition, changes ion fluxes, and activates ion-dependent metabolic processes (2–4). However, recent data indicate that rapid actions of 1α,25-$(OH)_2D_3$ can occur at very low concentrations (5–12) and with high cholecalciferol analog specificity (6, 7, 9–15), suggesting to us that the 1α,25-$(OH)_2D_3$ receptor could mediate rapid actions.

Rapid stimulation of cGMP by steroids (16–19) including 1α,25-$(OH)_2D_3$ (20) was reported in several tissues. Here we demonstrate a very rapid increase of intracellular cGMP after 1α,25-$(OH)_2D_3$ exposure in human cultured fibroblasts. By showing failure of this response in fibroblasts from subjects with hereditary defects in the 1α,25-$(OH)_2D_3$ receptor system we provide evidence that the rapid response is mediated by the 1α,25-$(OH)_2D_3$ receptor.

MATERIALS AND METHODS

Materials. Cell culture media were from Biofluids (Rockville, MD); defined fetal bovine serum was from HyClone (Logan, UT). 1α,25-$(OH)_2D_3$, 24,25-dihydroxycholecalciferol [24,25-$(OH)_2D_3$], and 25-hydroxycholecalciferol (25-$OH-D_3$) were gifts from M. Uskokovic (Hoffmann–La Roche). 1β,25-Dihydroxycholecalciferol [1β,25-$(OH)_2D_3$] was a gift from M. Holick and Rahul Ray (Boston University Medical School). Cholecalciferol, testosterone, dihydrotestosterone, and cGMP were from Sigma. ^{125}I-labeled succinyl-cGMP-tyrosine methyl ester (specific activity, >2000 μCi/1 μmol; 1 Ci = 37 GBq) was from Meloy Laboratories (Springfield, VA). Antiserum against cGMP (no. 33:5-6-81) was provided by Kevin Catt. All cholecalciferol analogs were tested for purity on reverse-phase high-performance liquid chromatography within a week before use (21).

Cell Culture. All studies were performed on human cultured skin fibroblasts. Three cell lines from patients with hereditary resistance to 1α,25-$(OH)_2D_3$ had been tested previously for 1α,25-$(OH)_2D_3$ receptor binding, for 1α,25-$(OH)_2D_3$ nuclear uptake, and for 1α,25-$(OH)_2D_3$ receptor elution from DNA-cellulose to characterize their defects in 1α,25-$(OH)_2D_3$ action (21–23) (Table 1). Cells were grown in Dulbecco's minimal essential medium/10% fetal bovine serum/0.2 mM glutamine/80 mg of gentamycin/0.1 μmol of insulin per liter. Cells were maintained in 150-cm^2 plastic tissue culture flasks (Costar, Cambridge, MA) at 37°C in an atmosphere of 5% CO_2/95% air, and they were subcultured every second week. For cGMP assays, cells were subcultured to 24-vial plates (Costar) and maintained for 24 hr in serum-free medium. Cell cultures from passages 12–30 were used at 75% confluency. Cells were tested for mycoplasma (American Type Culture Collection) with negative results.

Perturbation of Intracellular cGMP. Immediately before exposure to hormones cells were washed three times with medium A [Eagle's no. 2 medium without calcium, magnesium, sodium bicarbonate, and glutamine and with 25 mM Hepes and 2 mM manganese (pH 7.4)]. After removal of washing solution, cells were incubated with assay medium (medium A without manganese and with 1 mM calcium, 0.5 mM magnesium, 0.1 mg of isobutylmethylxanthine per ml, 0.3 g of bovine serum albumin per liter, and 500 units of aprotinin per liter). Different concentrations of steroids (each in triplicate wells) were also added to the assay medium. Each multivial plate included triplicate control wells for cGMP measurement without hormone, duplicates with 1 mM sodium nitroprusside, and others for protein measurement (Bio-Rad kit). Cells were incubated with assay medium at 37°C for 4 min with different concentrations of steroids (between 1 pM and 1 μM). For analysis of time course, cells were incubated with a steroid (0.1 μM) for 0.5–30 min, in duplicates, and compared to cells incubated simultaneously without the steroid. The reaction was stopped by removal of medium and addition of 0.5 ml of 90% 1-propanol. Cells were further incubated with the propanol at −20°C for 24 hr. Then, the supernates were lyophilized

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: 1α,25-$(OH)_2D_3$ (calcitriol), 1α,25-dihydroxycholecalciferol; 1β,25-$(OH)_2D_3$, 1β,25-dihydroxycholecalciferol; 25-$OH-D_3$, 25-hydroxycholecalciferol; 24,25-$(OH)_2D_3$, 24,25-dihydroxycholecalciferol; D_3, cholecalciferol.

Table 1. Characterization of abnormalities in cells from three subjects with hereditary resistance to 1α,25-$(OH)_2D_3$ (calcitriol)

Cell line	Calcitriol binding to soluble extract	Calcitriol uptake in nuclei	Calcitriol receptor elution from DNA-cellulose
10	Undetectable	NT*	NT*
2	Normal	Undetectable	Normal
7	Normal	Decreased	Abnormal

Cell lines are identified by kindred numbers, reported earlier (21–23).

*NT, not tested. Whenever hormone binding has been undetectable in soluble extracts, calcitriol uptake in nuclei and calcitriol receptor elution from DNA-cellulose have been undetectable {at least in part because the receptor cannot be labeled by 1α,25-$(OH)_2[^3H]D_3$} (21, 22).

under nitrogen and refrigerated until radioimmunoassay. Normal and 1α,25-$(OH)_2D_3$-resistant fibroblasts were always assayed in parallel.

Measurement of cGMP. The cGMP was measured in duplicate samples by acetylation and then by radioimmunoassay using a highly specific rabbit antiserum. The bound and free radioligand were separated by a second antibody technique. The lower limit of detection was 0.01 pmol/ml. Intraassay variation was 4%; interassay variation was 7%. Data were expressed as a percentage of control (no steroid added). Average basal cGMP among the six cell lines was 21 ± 9 pmol/mg of protein. A paired Student *t* test was used for statistical evaluation. Results are expressed as mean ± 1 SEM. Each experiment was repeated at least three times.

RESULTS

1α,25-$(OH)_2D_3$ Effect on Intracellular cGMP in Normal Fibroblasts. A 4-min incubation of normal cells with 1α,25-$(OH)_2D_3$ resulted in a dose-dependent increase in intracellular cGMP concentration (Fig. 1). The average half-maximal effect occurred at 1.8 ± 1.6 nM 1α,25-$(OH)_2D_3$. The maximal increase after 4 min of incubation with 0.1 μM 1α,25-$(OH)_2D_3$ varied among cell lines: line a, 315% ± 12%; line b, 280% ± 27%; line c, 155% ± 4%; line d, 360% ± 12% of the control level ($P < 0.01$ in each) (Fig. 1). The cGMP was significantly above baseline in three of four lines at 1 min ($P < 0.01$), reached a maximum at 6–8 min (225% ± 8%), and returned to control values within 30 min (Fig. 2). The cell

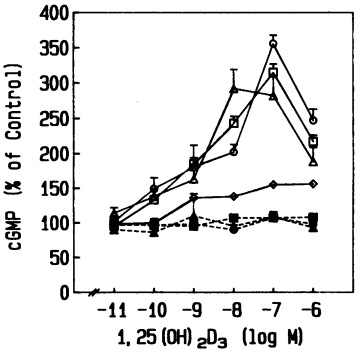

FIG. 1. Effect of various concentrations of 1α,25-$(OH)_2D_3$ on intracellular cGMP in normal human skin fibroblasts (open symbols: line a = □, line b = △, line c = ◇, line d = ○) and in skin fibroblasts from subjects with hereditary resistance to 1α,25-$(OH)_2D_3$ (closed symbols). The increase in cGMP was significant ($P < 0.01$) with 1α,25-$(OH)_2D_3$ at 1 nM or higher in three normal lines and at 0.1 μM or higher in the fourth normal line. Symbols represent the mean ± 1 SEM.

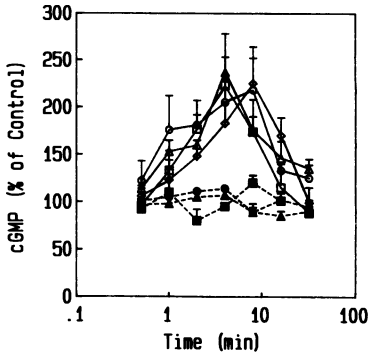

FIG. 2. Time course of (0.1 μM) 1α,25-$(OH)_2D_3$ effect on cGMP concentrations in normal human skin fibroblasts (same open symbols as Fig. 1) and in fibroblasts from subjects with hereditary resistance to calcitriol (closed symbols). The increase of cGMP was significant ($P < 0.01$) between 3 and 12 min in all normal cell lines, whereas there was no increase in any of the abnormal cell lines. Symbols represent the mean ± 1 SEM.

lines, tested for time course with the exception of line c, were from later passages than those tested for dose–response relation.

Calciferol Analog Specificity of cGMP Response in Normal Fibroblasts. In normal human skin fibroblasts, 24,25-$(OH)_2D_3$ stimulated cGMP only at high concentrations ($P < 0.001$). 1β,25-$(OH)_2D_3$ and 25-OH-D_3 caused a slight increase of cGMP at 1 μM concentration (the effect was not significant). Cholecalciferol caused no cGMP increase at any concentration tested (Fig. 3). All experiments in which a calciferol analog failed to stimulate cGMP involved positive responses of the same cells to 1,25-$(OH)_2D_3$ and sodium nitroprusside.

Intracellular cGMP Concentrations in 1α,25-$(OH)_2D_3$-Resistant Fibroblasts. Fibroblasts from subjects with hereditary resistance to 1α,25-$(OH)_2D_3$ showed no rise of intracellular cGMP at all doses and times of exposure to 1α,25-$(OH)_2D_3$ (Figs. 1 and 2).

Stimulation of Intracellular cGMP in Normal and in 1α,25-$(OH)_2D_3$-Resistant Fibroblasts by Other Agents. The average stimulation of cGMP by sodium nitroprusside was similar in four normal cell lines (4300% ± 1600%) and in three cell lines from subjects with hereditary resistance to 1α,25-$(OH)_2D_3$ (5100% ± 1200%) (Fig. 4).

We found a rapid increase of intracellular cGMP after dihydrotestosterone or testosterone exposure in normal and in 1α,25-$(OH)_2D_3$-resistant cells (Fig. 4). There were no

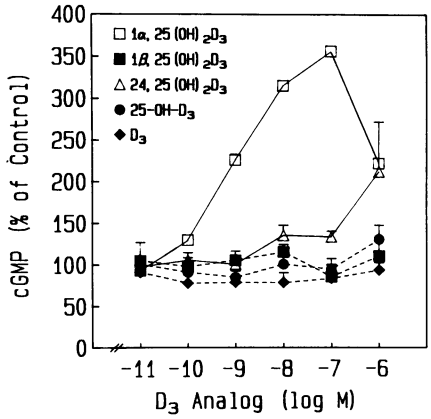

FIG. 3. Effects of 4-min incubation with cholecalciferol analogs on cGMP accumulation in one line of normal human fibroblasts. Symbols represent the mean ± 1 SEM of four experiments, each in triplicate.

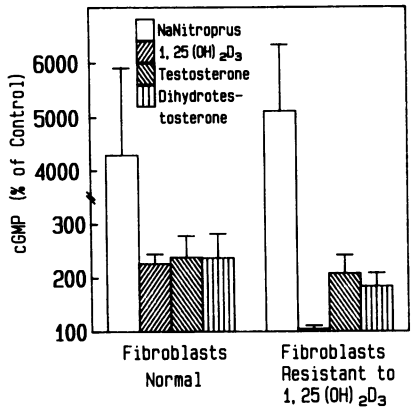

FIG. 4. Effects of four agents on intracellular cGMP in cells from normal controls (n = 4) and from subjects (n = 3) with hereditary resistance to 1α,25-$(OH)_2D_3$. The effects of sodium nitroprusside (1 mM), testosterone (0.1 μM), and dihydrotestosterone (0.1 μM) were similar in both groups. The effect of 1α,25-$(OH)_2D_3$ (0.1 μM) was different ($P < 0.001$) in the two groups. Symbols represent the mean ± 1 SEM.

statistically significant differences in cGMP rise after testosterone (0.1 μM) and dihydrotestosterone (0.1 μM) exposure between the normal cell lines and cell lines with hereditary resistance to 1α,25-$(OH)_2D_3$ (Fig. 4).

DISCUSSION

We have found that 1α,25-$(OH)_2D_3$ caused a significant and rapid increase of intracellular cGMP concentration in normal human skin fibroblasts. This extends prior reports that 1α,25-$(OH)_2D_3$ could raise cGMP within 10–120 min in kidney (24), duodenum (24, 25), and bone (26). In skin fibroblasts the rise of cGMP was detectable as early as 1 min after addition of 1,25-$(OH)_2D_3$.

The inclusion of a phosphodiesterase inhibitor (isobutylmethylxanthine) suggests that the increase of cGMP reflects increased synthesis rather than decreased degradation.

This effect of 1α,25-$(OH)_2D_3$ occurred within 1 min, too rapidly to be mediated by genomic effects (1). Other rapid (significant within <30 min) actions of 1α,25-$(OH)_2D_3$ have been described, including effects on adenylate cyclase in the kidney (11) and parathyroid (27); on transmucosal calcium transport in duodenum (7, 8, 28, 29); on cytosolic calcium in the liver (9), parathyroid (14), epidermis (13), and osteoblastic cells (12, 30); on membrane potential in cartilage and renal proximal tubule (6); and on phospholipid metabolism in multiple tissues (5, 31, 32). The transduction mechanisms for these rapid actions of 1α,25-$(OH)_2D_3$ have not been established.

We observed an elevation of cGMP concentration at low concentrations of calcitriol, suggesting the involvement of a protein capable of binding tightly to this hormone. The selectivity of cGMP response for 1α,25-$(OH)_2D_3$ (preference against other cholecalciferol analogs and also stereospecificity with regard to 1-hydroxyl group) suggested mediation by a typical 1α,25-$(OH)_2D_3$ receptor. Finally, the unresponsiveness of cells from subjects with hereditary resistance to 1α,25-$(OH)_2D_3$ strongly supported the notion that this rapid action was mediated by a molecule similar or identical to the one that modulates genomic effects of 1α,25-$(OH)_2D_3$.

The normal cGMP response of the 1α,25-$(OH)_2D_3$-resistant cells to sodium nitroprusside or testosterone suggested that there was no general disorder in these cells leading to unresponsiveness of cGMP to multiple regulatory stimuli.

The three 1α,25-$(OH)_2D_3$-resistant cell lines represented three types of receptor defect: one had undetectable hormone binding, another had normal hormone binding to soluble extracts but undetectable nuclear uptake of hormone in intact cells, and one showed normal hormone binding but a decreased affinity of the receptor for DNA. All three types of receptor defect resulted in unresponsiveness to a rapid action (cGMP elevation) of 1α,25-$(OH)_2D_3$. On the basis of this finding at least three receptor functions are necessary for this rapid action of 1α,25-$(OH)_2D_3$.

Further studies will be required to establish the structure and subcellular location of the receptors mediating this, and perhaps other, rapid action of 1α,25-$(OH)_2D_3$, the most proximal effects of the receptors, and the mechanism and location for receptor-mediated increase of cGMP concentration.

Our study establishes that the same defective genes that disturb the classical 1α,25-$(OH)_2D_3$ genomic mechanism cause analogous interference with a rapid, presumably nongenomic, effect of calcitriol. Therefore, we conclude that the receptor molecules that can mediate a rapid effect of 1α,25-$(OH)_2D_3$ are identical or closely related to the receptors that mediate genomic effects.

1. Duval, D., Durant, S. & Homo-Delarche, F. (1983) *Biochim. Biophys. Acta* **737**, 409–442.
2. Kurnik, B. R. C., Huskey, M. & Hruska, D. A. (1987) *Biochim. Biophys. Acta* **917** 81–85.
3. Majewska, M. D., Harrison, N. L., Schwartz, R. D., Barker, J. L. & Paul, S. M. (1986) *Science* **232**, 1004–1007.
4. Nabekura, J., Oomura, Y., Minami, T., Mizuno, Y. & Fukuda, A. (1986) *Science* **233**, 226–228.
5. Kurnik, B. R. C., Huskey, M., Hagerty, D. & Hruska, K. A. (1986) *Biochim. Biophys. Acta* **858**, 47–55.
6. Edelman, A., Garabedian, M. & Anagnostopoulos, T. (1986) *J. Membr. Biol.* **90**, 137–143.
7. Nemere, I., Yoshimoto, Y. & Norman, A. (1984) *Endocrinology* **115**, 1476–1483.
8. Yoshimoto, Y., Nemere, I. & Norman, A. (1986) *Endocrinology* **118**, 2300–2304.
9. Baran, D. T. & Milne, M. L. (1986) *J. Clin. Invest.* **77**, 1622–1626.
10. Karsenty, G., Lacour, B., Ulmann, A., Pierandrei, E. & Drueke, T. (1985) *Am. J. Physiol.* **248**, G40–G45.
11. Cloix, J. F., d'Herbigny, E. & Ulmann, A. (1980) *J. Biol. Chem.* **255**, 11280–11283.
12. Oshima, J., Watanabe, M., Hirosumi, J. & Orimo, H. (1987) *Biochem. Biophys. Res. Commun.* **145**, 956–960.
13. MacLaughlin, J. A., Cantley, L. C., Chahwala, S. B. & Holick, M. F. (1987) *J. Bone Miner. Res.* **2**, Suppl. 76 (abstr.).
14. Sugimoto, T., Ritter, C., Ried, I., Morrisey, J. & Slatopolsky, E. (1987) *J. Bone Miner. Res.* **2**, Suppl. 79 (abstr.).
15. Dufy, B., Vincent, J. D., Fleury, H., Du Pasquier, P., Gourdji, D. & Tixier-Vidal, A. (1979) *Science* **204**, 509–511.
16. Vesely, D. L. (1979) *Proc. Natl. Acad. Sci. USA 76*, 3491–3494.
17. Vesely, D. L. & Hill, D. (1980) *Endocrinology* **107**, 2104–2109.
18. Vesely, D. L. (1980) *J. Pharmacol. Exp. Ther.* **214**, 561–566.
19. Whalen, R. E. & Lauber, A. H. (1986) *Neurosci. Biobehav. Rev.* **10**, 47–53.
20. Juan, D. & Vesely, D. L. (1984) in *Endocrine Control of Bone and Calcium Metabolism*, eds. Cohn, D. V., Fujita, T., Potts, J. T., Jr., & Talmage, R. V. (Elsevier, New York), pp. 372–375.
21. Gamblin, G. T., Liberman, U. A., Eil, C., Down, R. W., Jr., DeGrange, D. A. & Marx, S. J. (1985) *J. Clin. Invest.* **75**, 954–960.
22. Liberman, U. A., Eil, C. & Marx, S. J. (1986) *J. Clin. Endocrinol. Metab.* **62**, 122–126.
23. Bliziotes, M., Yergey, A. L. Nanes, M. S., Muenzer, J., Begley, M. G., Vieira, N. E., Kher, K. K., Brandi, M. L. & Marx, S. J. (1988) *J. Clin. Endocrinol. Metab.*, in press.
24. Vesely, D. L. & Juan, D. (1984) *Am. J. Physiol.* **246**, E115–E120.
25. Guillemant, J. & Guillemant, S. (1980) *Biochem. Biophys. Res. Commun.* **93**, 906–911.
26. Howard, G. A. (1985) in *Normal and Abnormal Bone Growth:*

Basic and Clinical Research, eds. Dixon, A. D. & Sarnat, G. (Liss, New York), pp. 67–76.
27. Cloix, J. F., Ulmann, A., Monet, J. D. & Funck-Brentano, J. L. (1981) *Clin. Sci.* **60,** 339–341.
28. Nemere, I. & Norman, A. (1986) *Endocrinology* **119,** 1406–1408.
29. Bikle, D. D., Zolock, D. T., Morrisey, R. L. & Herman, R. H. (1978) *J. Biol. Chem.* **253,** 484–488.
30. Lieberherr, M. (1987) *J. Biol. Chem.* **262,** 13168–13173.
31. Kreutter, D., Matsumoto, T., Peckham, R., Zawalich, K., Wen, W. H., Zolock, D. T. & Rasmussen, H. (1983) *J. Biol. Chem.* **258,** 4977–4981.
32. Matsumoto, T., Fontaine, O. & Rasmussen, H. (1981) *J. Biol. Chem.* **256,** 3354–3360.