Effect of 1,25-dihydroxyvitamin D₃ on cyclic AMP responses to hormones in clonal osteogenic sarcoma cells

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The effect of 1,25-dihydroxyvitamin D₃ on adenylate cyclase responsiveness was studied in the clonal osteogenic sarcoma cell line, UMR 106-06, which responds to several bone active hormones. 1,25-dihydroxyvitamin D₃ treatment had no consistent effect on basal formation of cyclic AMP in intact cells, but the responses to parathyroid hormone, isoproterenol, prostaglandin E₂, salmon calcitonin and the plant diterpene, forskolin, were all attenuated, by up to 90%. The effect of 1,25-dihydroxyvitamin D₃ was dose-dependent, with half-maximal effectiveness at 0.1 nM, and required 48 h treatment of cells before it became apparent. The relative potencies of other vitamin D₃ compounds correlated closely with their relative affinities for the 1,25-dihydroxyvitamin D₃ receptor and their biological activities in other systems. 1,25-dihydroxyvitamin D₃ treatment had no effect on the kinetics of labelled calcitonin binding to UMR 106-06 cells. Furthermore, the fact that such a range of hormones was affected made a receptor mediated mechanism unlikely. Nucleotide stimulatory (N_s) unit activity was assayed after 1,25-dihydroxyvitamin D₃ treatment and found to be unchanged. Islet activating protein, an inhibitor of nucleotide inhibitory unit (N_i) activity, failed to modify the 1,25-dihydroxyvitamin D₃ effect. Thus the effect of 1,25-dihydroxyvitamin D₃ appeared to be exerted beyond hormone receptor and nucleotide regulatory components of the adenylate cyclase complex. It is concluded that 1,25-dihydroxyvitamin D₃ attenuates adenylate cyclase response to hormones by a direct or indirect action on the catalytic component of adenylate cyclase.

INTRODUCTION

Receptors for 1,25(OH)₂D₃ have been identified in many cell types (for review see Eisman, 1983). Although in many respects 1,25(OH)₂D₃ behaves as a steroid hormone, in some targets its physiological actions are similar to those of hormones which act through cyclic AMP. This is the case in bone, in which both 1,25(OH)₂D₃ and PTH promote bone resorption (Raisz *et al.*, 1972). Whereas PTH increases cyclic AMP formation as an important early event in its action (Chase *et al.*, 1969), 1,25(OH)₂D₃ has no known direct effect upon cyclic AMP generation (Wong *et al.*, 1977). Their common physiological effects prompted studies of interactions between these two hormones in their early effects on cells. Wong *et al.*, (1977) showed that treatment of isolated bone cells with 1,25(OH)₂D₃ resulted in refractoriness to stimulation of cyclic AMP formation by PTH, but not by calcitonin. The refractoriness was evident within 6 h of treatment, and complete within 24 h. This was confirmed in later experiments in which refractoriness to PGE₂ was also demonstrated after 1,25(OH)₂D₃ treatment (Kent *et al.*, 1980), and other workers noted refractoriness to PTH in 1,25(OH)₂D₃-treated bone cells (Crowell *et al.*, 1981). Previous data in this area had been conflicting, in that 1,25(OH)₂D₃ had been found by some to increase cyclic AMP responsiveness (Mahgoub & Sheppard, 1975) and by others to have no effect (Kakuta *et al.*, 1975; Peck & Dowling, 1976; Herrman-Erlee *et al.*, 1980; Lieberherr *et al.*, 1980). Recent observations in clonal osteogenic sarcoma cells (Catherwood, 1985) and in a human

monocyte line (Rubin & Catherwood, 1984) revealed that 1,25(OH)₂D₃ treatment of those cells resulted in diminished responsiveness to PTH and to isoproterenol respectively.

In the present work we have investigated the effect of 1,25(OH)₂D₃ treatment on hormonal responses in a clonal osteogenic sarcoma cell line of rat origin, UMR 106-06 (Forrest *et al.*, 1985). This line, a subclone derived from UMR 106 (Partridge *et al.*, 1983), responds to PTH, PGE₂, calcitonin and isoproterenol with an increase in cyclic AMP. The results show that 1,25(OH)₂D₃ treatment leads to refractoriness to each of those hormones, and that this effect of 1,25(OH)₂D₃ is likely to be mediated through its receptor. Furthermore, the mechanism of action of 1,25(OH)₂D₃ has been investigated, and the lack of involvement of nucleotide regulatory subunits is consistent with an action of the steroid beyond this component of the adenylate cyclase complex.

MATERIALS AND METHODS

Hormones and chemicals

PTH, synthetic human parathyroid hormone, hPTH(1–34), was obtained from Beckman, Palo Alto, CA, U.S.A. Vitamin D₃ compounds were generously provided by Dr. M. Uskokovic, Hoffman-LaRoche, Nutley, NJ, U.S.A. PGE₂ was purchased from the Upjohn Co., Kalamazoo, MI, U.S.A. Synthetic salmon calcitonin (4700 units/mg) was a gift from Dr. R. C. Orlowski, Armour Pharmaceutical Co., Kankakee, IL, U.S.A.

Abbreviations used: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃ (calcitriol); PTH, parathyroid hormone; IAP, islet activating protein; PGE₂, prostaglandin E₂; N_s, nucleotide stimulatory units; N_i, nucleotide inhibitory units.

Bovine serum albumin was from Armour Pharmaceutical Co. Foetal calf serum and culture medium were obtained from Flow Laboratories. 3-Isobutyl-1-methylxanthine was obtained from Sigma. All nucleotides used were obtained from Boehringer-Mannheim. [2,8-³H]Adenine (12.8 Ci/mmol) was obtained from New England Nuclear, and [α -³²P]ATP, cyclic [³²P] AMP, cyclic [³H] AMP and Na¹²⁵I from The Radiochemical Centre, Amersham, Bucks., U.K. Forskolin was obtained from Calbiochem, La Jolla, CA, U.S.A. Isoproterenol was from Breon Laboratories, New York, NY, U.S.A. IAP, the active component of *Bordetella pertussis* toxin, was a gift to Professor R. G. Larkins from Dr. Yajima, Shiga, Japan. All other chemicals were of reagent grade and obtained from standard suppliers.

Cell culture of UMR cells

UMR 106-06 cells (Forrest *et al.*, 1985) were grown in 75 cm² plastic culture flasks (Miles Laboratories, Naperville, IL, U.S.A.) in modified Eagle's minimal essential medium containing 10% (v/v) foetal calf serum, gentamycin (80 mg/l) and minocyclin (1 mg/l) and were incubated at 37 °C in CO₂/air (1:19). Replicate subcultures were performed, and cells were harvested with 0.0125% trypsin and 0.5 mM-disodium EDTA in phosphate-buffered saline, pH 7.4. Cells were seeded into 12-well tissue culture dishes (4.5 cm²/well; Flow Laboratories) at approx. 5 × 10⁴ cells/well. After the cells had become attached to the wells overnight, vitamin D compounds dissolved in ethanol or vehicle were added in fresh medium containing 2% (v/v) foetal calf serum treated with charcoal, which decreases concentrations of endogenous 1,25(OH)₂D₃ by 90% (Eisman *et al.*, 1980). The final concentration of ethanol was 0.02% (v/v). Medium was changed every second day. For treatment with IAP, cells were incubated with fresh medium containing 0.1% (w/v) bovine serum albumin and 1.6 × 10⁻¹⁰ M-IAP at 37 °C for 16 h before the determination of cyclic AMP generation. Cells were counted in a Coulter model D N counter, and in each experiment cell numbers were obtained for control and 1,25(OH)₂D₃-treated cultures by counting quadruplicate wells.

Cyclic AMP generation in intact cells

Cells were incubated with [³H]adenine (1 μ Ci/well) for 2 h at 37 °C in 500 μ l of fresh culture medium containing 0.1% (w/v) bovine serum albumin. The medium was aspirated, and cells were washed twice with phosphate-buffered saline, pH 7.4. Cells were preincubated in 900 μ l of culture medium with 1 mM-3-isobutyl-1-methylxanthine for 20 min at 37 °C. A 100 μ l sample of medium containing agonist was added for additional 10 min. The reaction was stopped by removing the medium and adding 100 μ l of 1.2 M-trichloroacetic acid followed by 800 μ l of a mixture of 5 mM-adenine, AMP, ADP, ATP and cyclic AMP; 100 μ l of cyclic [³²P]AMP (5000 c.p.m.) was added to each well to calculate recoveries. These samples were then subjected to sequential chromatography on Dowex 50W-X4 (0.7 cm × 4 cm column, 200–400 mesh; Bio-Rad) and alumina to purify the cyclic [³H]AMP formed in the cells (Salomon *et al.*, 1974; Michelangeli *et al.*, 1984). Radioactivity was counted in Beckman Ready-Solve EP scintillation cocktail in a Beckman scintillation counter, model LS 2800. Data were

expressed as the amount of cyclic [³H]AMP generated/10⁶ cells.

Binding of labelled salmon calcitonin

¹²⁵I-labelled salmon calcitonin binding was assayed as described previously for breast cancer cells (Lamp *et al.*, 1981). Cells subcultured in wells were washed with phosphate-buffered saline, pH 7.4. Synthetic salmon calcitonin was labelled with Na¹²⁵I as previously described (Hunt *et al.*, 1977) using 2 μ g of chloramine-T for 10 s. Purified labelled hormone was stored in acetone/1% acetic acid (1:4, v/v) in small samples. Incubations were carried out in a total volume of 500 μ l [400 μ l of medium containing 0.1% (w/v) bovine serum albumin, 50 μ l of ¹²⁵I-labelled salmon calcitonin (10⁵ c.p.m./well) and 50 μ l of varying amounts of unlabelled salmon calcitonin] at 37 °C for 60 min with continuous agitation. The medium was then aspirated and the cells were washed twice with phosphate-buffered saline, pH 7.4 at 4 °C. The reaction was terminated by solubilizing cells with 100 μ l of 0.1 M-NaOH, and their radioactivity was measured in a Packard Auto-Gamma scintillation spectrometer, model 5110.

Preparation of cyc⁻ S49 membranes

Preparation of cyc⁻ S49 membranes, extraction of UMR 106-06 N-component and reconstitution assay were performed as described (Ross *et al.*, 1977; Iyengar *et al.*, 1981; Rich *et al.*, 1984), with minor modifications. Cyc⁻ S49 cells (clone 94.15.1), obtained from the University of California Cell Repository, San Francisco, CA, U.S.A., were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal calf serum at 37 °C in CO₂/air (1:19) in 175 cm² plastic culture flasks (Nunc, Roskilde, Denmark). Cells were harvested at low speed (1000 rev./min, 10 min), and washed with phosphate-buffered saline, pH 7.4. Cells resuspended in 150 mM-NaCl/2 mM-MgCl₂/1 mM-EDTA/1 mM-2-mercaptoethanol/20 mM-Hepes, pH 7.4, were then lysed by nitrogen cavitation [2.76 MPa (400 lbf/in²) for 20 min at 4 °C] in a Parr nitrogen disruption apparatus (Parr Instrument Co., Moline, IL, U.S.A.), and centrifuged at 750 g for 5 min. The supernatants were then centrifuged at 43000 g for 20 min at 4 °C. The pellets were resuspended in a mixture of 10% (w/v) sucrose/2 mM-MgCl₂/1 mM-EDTA/1 mM-2-mercaptoethanol/10% (v/v) glycerol/25 mM-Hepes/NaOH, pH 8.0, at a protein concentration of 2 mg/ml and stored at -70 °C until use.

Extraction of UMR 106-06 cell membrane nucleotide regulatory activity

UMR 106-06 cell membranes with or without 1,25(OH)₂D₃ pretreatment were prepared according to the procedure used for cyc⁻ S49 cell membranes. The pellets from the 43000 g centrifugation were resuspended and stirred in 50 mM-NaCl/1 mM-EDTA/1 mM-2-mercaptoethanol/10% (w/v) sucrose/1% (w/v) sodium cholate/25 mM-Hepes/NaOH, pH 8.0, at 4 °C for 30 min. The suspension was centrifuged at 100000 g for 60 min at 4 °C. Protein concentrations of the supernatants of both treatment groups were adjusted to 500 μ g/ml with the same buffer. These supernatants were incubated at room temperature for 20 min to inactivate their adenylate cyclase catalytic unit activity.

Table 1. Effect of 1,25(OH)₂D₃ treatment on cyclic AMP generation in response to various agonists in UMR 106-06 cells

UMR 106-06 cells were treated with 10 nM-1,25(OH)₂D₃ for 5 days. After the treatment, [³H]cyclic AMP accumulation was determined in cells prelabelled with [³H]adenine, and incubated with 2.7 nM-PTH, 30 nM-PGE₂, 3 nM-salmon calcitonin, 10 μM-isoproterenol or 1 μM-forskolin for 10 min. Values are means ± S.E.M. for quadruplicate assays; N.S., not significant. Cell counts: for control, (1.81 ± 0.43) × 10⁵; for 1,25(OH)₂D₃-treated, (1.74 ± 0.44) × 10⁵ (means ± S.E.M., *n* = 4, N.S.).

Conditions	[³ H]cyclic AMP generated (c.p.m./10 ⁶ cells)		Significance versus appropriate control
	Control	1,25(OH) ₂ D ₃ (10 nM)	
Basal	293 ± 132	155 ± 69	N.S.
PTH	54547 ± 1464	8126 ± 132	<i>P</i> < 0.001
PGE ₂	13122 ± 536	5678 ± 161	<i>P</i> < 0.001
Salmon calcitonin	26906 ± 635	12839 ± 253	<i>P</i> < 0.001
Isoproterenol	9707 ± 409	2029 ± 103	<i>P</i> < 0.001
Forskolin	11950 ± 260	6667 ± 448	<i>P</i> < 0.001

Reconstitution assays of UMR cell membrane nucleotide regulatory unit stimulatory (N_s) activity

Reconstitution was carried out by incubating cyc⁻ S49 membranes with diluted cholate extract of UMR 106-06 cell membranes at 32.5 °C for 10 min in the presence of 25 mM-Hepes/NaOH (pH 8.0)/2 mM-MgCl₂/1 mM-EDTA/0.5 mM-ATP/0.1 mM-GTP/0.25% cholate. Reconstituted mixture (20 μl) was incubated with 30 μl of adenylate cyclase assay mixture containing 0.1 mM-[α-³²P]ATP (10⁶ c.p.m./tube), 33 mM-Hepes/NaOH pH 8.0, 1.3 mM-EDTA, 2 mM-MgCl₂, 0.13 mM-GTP, 0.13 mM-cyclic AMP, 27 mM-phosphocreatine, 0.3 mg of creatine kinase/ml, 0.03 mg of myokinase/ml, and with or without 180 μM-isoproterenol at 32.5 °C for 30 min with continuous agitation. The final concentration of cholate was 0.1% (w/v). The reaction was stopped by adding 100 μl of 40 mM-ATP and boiling for 3 min. The [³²P]cyclic AMP formed was separated according to the procedure described in the method for cyclic AMP generation in intact cells, using [³H]cyclic AMP (5000 c.p.m./tube) to calculate recoveries (Salomon *et al.*, 1974). The protein concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

RESULTS

Effect of 1,25(OH)₂D₃ treatment on cyclic AMP responses in UMR 106-06 cells

When UMR 106-06 cells in culture were treated with 1,25(OH)₂D₃ (10 nM) for 5 days, and the ability of cells to generate cyclic AMP in response to various agents was measured, the response to all agonists was diminished (Table 1). This was observed not only with hormones, but also with the plant diterpene, forskolin. The basal level of cyclic AMP was not consistently different in control and 1,25(OH)₂D₃-treated cultures, nor did treatment alter cell numbers. Furthermore, in none of the following experiments did 1,25(OH)₂D₃ affect cell numbers, up to a concentration of 10 nM. Treatment of cells with as much as 100 nM-1,25(OH)₂D₃ resulted in decreases of up to 20% in cell numbers (results not shown).

Effect of vitamin D compounds on the cyclic AMP response to PTH in UMR 106-06 cells

Treatment of UMR 106-06 cells for 5 days with increasing concentrations of 1,25(OH)₂D₃ resulted in a dose-dependent decrease in the cyclic AMP response to PTH (Fig. 1). The half-maximally effective concentration of 1,25(OH)₂D₃ for this response was 0.1 nM. 24,24-Difluoro-1,25(OH)₂D₃ was slightly more effective than 1,25(OH)₂D₃ itself, with half maximal effectiveness at 0.03 nM. This is consistent with its known greater biological activity upon chick duodenum (Corradino *et al.*, 1980), and approximately equal affinity with that of 1,25(OH)₂D₃ for the receptor in breast cancer cells

Fig. 1. Effect of vitamin D₃ compounds on cyclic AMP generation in response to PTH in UMR 106-06 cells

UMR 106-06 cells were treated with various concentrations of vitamin D₃ compounds for 5 days as follows: ●, 24,24-difluoro-1,25(OH)₂D₃; ○, 1,25(OH)₂D₃; □, 1,24,25(OH)₃D₃; △, 25(OH)₂D₃; ▲, 24,25(OH)₂D₃. After the treatment, [³H]cyclic AMP accumulation was determined in cells prelabelled with [³H]adenine as described in the Materials and methods section. Values are expressed as percentage inhibition of maximum cyclic AMP accumulation, which was 23499 ± 533 c.p.m./10⁶ cells treated with PTH (2.7 nM) without vitamin D₃ compounds. Points are means ± S.E.M. for triplicate assays.

Fig. 2. Time course of response to $1,25(\text{OH})_2\text{D}_3$ in UMR 106-06 cells

UMR 106-06 cells were treated with 10 nM - $1,25(\text{OH})_2\text{D}_3$ for the various times indicated. After the treatment, $[^3\text{H}]$ cyclic AMP accumulation was determined in cells prelabelled with $[^3\text{H}]$ adenine, and treated for 10 min with $\circ$, 2.7 nM -PTH; $\bullet$, 30 nM - PGE_2 ; $\square$, 3 nM -salmon calcitonin; $\blacktriangle$, $10 \mu\text{M}$ -isoproterenol; $\triangle$, $1 \mu\text{M}$ -forskolin, for 10 min. $[^3\text{H}]$ Cyclic AMP accumulation was determined. Values are expressed as percentage of maximum cyclic AMP accumulation, and are means $\pm$ S.E.M. for quadruplicate assays.

(Eisman *et al.*, 1983). On the other hand, the difluoro compound was found to be less effective than $1,25(\text{OH})_2\text{D}_3$ in promoting bone resorption (Stern *et al.*,

1981). Subtle differences in biological activity of the difluoro compound in different assay systems are thought to be related to its resistance to metabolism in the C-24 position (Eisman *et al.*, 1983). $1,24,25(\text{OH})_3\text{D}_3$ was somewhat less effective, and $24,25(\text{OH})_2\text{D}_3$ as well as $25(\text{OH})\text{D}_3$ at least 100 times less effective, than $1,25(\text{OH})_2\text{D}_3$ in reducing responsiveness to PTH.

Time course of response to $1,25(\text{OH})_2\text{D}_3$

When UMR 106-06 cells were treated with $1,25(\text{OH})_2\text{D}_3$ (10 nM) and cyclic AMP responses to the several hormones and forskolin were measured at intervals thereafter, decreased responsiveness to both PTH and isoproterenol were noted first on day 2, and became maximum by day 4 (Fig. 2). Diminished responses to salmon calcitonin, PGE_2 and forskolin only became apparent by day 4. In no case was any significant attenuation of hormone response noted within 24 h, which contrasts with previous observations in mixed bone cells in which effects were usually complete by 24 h (Wong *et al.*, 1977; Kent *et al.*, 1980; Crowell *et al.*, 1981).

Effect of $1,25(\text{OH})_2\text{D}_3$ on responsiveness of UMR 106-06 cells to hormones

Treatment of UMR 106-06 cells with $1,25(\text{OH})_2\text{D}_3$ (10 nM) for 5 days resulted in decreased cyclic AMP generation in response to maximum hormone concentrations (Fig. 3). In the case of PTH this was consistently associated with some decrease in sensitivity, but no such effect was obtained with calcitonin or PGE_2 . In the experiment of Fig. 3, $1,25(\text{OH})_2\text{D}_3$ treatment increased the half maximally effective concentration of PTH from 0.3 nM to 5.0 nM . For salmon calcitonin this parameter

Fig. 3. Effect of $1,25(\text{OH})_2\text{D}_3$ on dose-response of UMR 106-06 cells to hormones

UMR 106-06 cells were treated with 10 nM - $1,25(\text{OH})_2\text{D}_3$ for 5 days. After the treatment, $[^3\text{H}]$ cyclic AMP accumulation was determined in cells prelabelled with $[^3\text{H}]$ adenine, and incubated with various concentrations of PTH (left), salmon calcitonin (middle) or PGE_2 (right) for 10 min. $\circ$, Control cells, $\bullet$, cells treated with $1,25(\text{OH})_2\text{D}_3$. Values are means $\pm$ S.E.M. for triplicate determinations.

Fig. 4. Effect of 1,25(OH)₂D₃ treatment on binding of labelled calcitonin to UMR 106-06 cells

UMR 106-06 cells were treated with 10 nM-1,25(OH)₂D₃ (●) or vehicle (○) for 5 days. After the treatment, cells were incubated with ¹²⁵I-salmon calcitonin (10⁵ c.p.m./well) and various amounts of unlabelled hormone for 60 min at 37 °C. Cell numbers per well were $(4.84 \pm 0.65) \times 10^5$ (control) and $(4.83 \pm 0.43) \times 10^5$ [1,25(OH)₂D₃ treated]. Specific binding was determined as described in the Materials and methods section. Points are means for triplicate assays. The inset shows Scatchard plots of ¹²⁵I-salmon calcitonin binding in this experiment. The K_d values of untreated and 1,25(OH)₂D₃-treated cells were 470 and 480 nM respectively.

remained unchanged at 0.08 nM, and likewise for PGE₂ at 200 nM. In the latter case, although a plateau response was not reached in the experiment of Fig. 3, higher concentrations of PGE₂ produced no greater cyclic AMP responses in other experiments.

Effects of 1,25(OH)₂D₃ treatment on binding of labelled calcitonin to UMR 106-06 cells

Since the UMR 106-06 cells possess specific receptors for calcitonin (Forrest *et al.*, 1985), and since receptor studies with calcitonin can be carried out more reliably than with PTH, the binding of biologically active labelled salmon calcitonin to intact UMR 106-06 cells was measured in cells with or without treatment with

1,25(OH)₂D₃ for 5 days. The results (Fig. 4) show that treatment had no effect on the binding of calcitonin to the cells, indicating that any effect upon the cyclic AMP response to calcitonin took place beyond the hormone receptor.

Effect of treatment of UMR 106-06 cells with IAP on the response to 1,25(OH)₂D₃

It seemed likely that the effect of 1,25(OH)₂D₃ on hormone response occurred at some site beyond hormone-receptor interaction. Adenylate cyclase activation requires the participation of nucleotide stimulatory (N_s) and nucleotide inhibitory (N_i) units. Stimulation results from activation of N_s (Cooper *et al.*, 1979), and inhibition is mediated by N_i (Hildebrandt *et al.*, 1982). IAP, the active protein of *Bordetella pertussis* toxin, has been used effectively as an inhibitor of N_i unit activity (Katada & Ui, 1982; Bokoch *et al.*, 1983; Michelangeli *et al.*, 1984; Asano *et al.*, 1984). Since it was possible that 1,25(OH)₂D₃ treatment led to enhanced N_i unit activity, thereby decreasing cyclic AMP response, experiments were carried out to test this possibility. UMR 106-06 cells were treated with IAP (1.6×10^{-10} M), and cyclic AMP responses to PTH were measured in cells with or without 5 days pre-treatment with 1,25(OH)₂D₃. IAP treatment had no significant effect on the ability of 1,25(OH)₂D₃ to impair responsiveness to PTH (Table 2). The conditions used for treatment with IAP were those which we had shown in other cells to be effective in blocking N_i unit activity (Michelangeli *et al.*, 1984).

Effect of 1,25(OH)₂D₃ treatment of UMR 106-06 cells on N_s unit content

An alternative possible explanation for reduced hormone responsiveness was a decrease in the number of N_s units as a result of treatment with 1,25(OH)₂D₃. This was assessed by assaying detergent extracts of UMR 106-06 cells [with or without 1,25(OH)₂D₃ treatment] for their content of N_s, using the ability of the extracts to confer adenylate cyclase responsiveness on membrane preparation of cyc⁻S49 mouse lymphoma cells, which are deficient in N_s unit activity (Bourne *et al.*, 1975). The results (Table 3) showed that 1,25(OH)₂D₃ treatment did not lead to any loss of total N_s unit activity from UMR 106-06 cells as measured in the reconstitution assay. Under these conditions no adenylate cyclase activity or isoproterenol response could be detected in cyc⁻S49 membranes without addition of cholate extracts containing N_s unit activity.

Table 2. Lack of effect of pertussis toxin (IAP) on 1,25(OH)₂D₃ response in UMR 106-06 cells

UMR 106-06 cells were treated with 10 nM-1,25(OH)₂D₃ for 5 days, and IAP treatment was carried out for 16 h. [³H]Cyclic AMP accumulation was determined in cells prelabelled with [³H]adenine, and incubated with 2.7 nM-PTH for 10 min. Values are means $\pm$ S.E.M. for quadruplicate assays. Significance: *P < 0.005, **P < 0.001 versus control [untreated with 1,25(OH)₂D₃].

Conditions	[³ H]Cyclic AMP generated (c.p.m./10 ⁶ cells)			
	No treatment		IAP (1.6×10^{-10} M)	
	Control	1,25(OH) ₂ D ₃ (10 nM)	Control	1,25(OH) ₂ D ₃ (10 nM)
Basal	277 $\pm$ 19	194 $\pm$ 2*	329 $\pm$ 12	224 $\pm$ 11*
PTH	16687 $\pm$ 317	5922 $\pm$ 183**	18771 $\pm$ 738	6100 $\pm$ 93**

Table 3. Effect of 1,25(OH)₂D₃ on N_s activity in UMR 106-06 cells

UMR 106-06 cells were treated with 10 nM-1,25(OH)₂D₃ or vehicle for 5 days. Extraction and assay of N_s unit activity by reconstitution of isoproterenol-stimulated adenylate cyclase activity in cyc⁻S49 cells membranes were performed as described in the Materials and methods section. Values are means ± S.E.M. for triplicate assays. Significance: *No significant difference from control [untreated with 1,25(OH)₂D₃]; **P < 0.001 versus appropriate basal value.

Conditions	Cyclic AMP generated (pmol/min per mg of cholate extract)	
	Control	1,25(OH) ₂ D ₃ (10 nM)
Basal	28.8 ± 1.2	30.5 ± 1.7*
Isoproterenol (100 μM)	86.1 ± 2.9**	79.9 ± 3.1**

DISCUSSION

The ability of 1,25(OH)₂D₃ treatment to give rise to 'heterologous desensitization' to PTH and PGE₂ in mixed bone cell cultures has been noted previously (Wong *et al.*, 1977; Kent *et al.*, 1980), but there has been no study of the specificity of this effect or its mechanism. The experiments reported here in a clonal line of osteoblast phenotype show that the vitamin D hormone exerts its effects on cyclic AMP responsiveness whatever the agent used to enhance cyclic AMP formation. The experiments were carried out under conditions of maximum inhibition of cyclic nucleotide phosphodiesterase, so effects of 1,25(OH)₂D₃ on that enzyme cannot contribute to the results obtained. The time taken for appearance of reduced hormone response was appreciably longer than that for mixed bone cell cultures, in which effects were seen as early as 6 h after starting treatment (Wong *et al.*, 1977). It is clearly shown in this work that the response to 1,25(OH)₂D₃ is a sensitive one, and furthermore that the relative effectiveness of various metabolites and analogues of vitamin D reflects their abilities to compete for binding to the 1,25(OH)₂D₃ receptor (Eisman, 1983). Thus it seems likely that the effect of 1,25(OH)₂D₃ is mediated through the receptor for this steroid.

It is not yet possible to identify the site of action of 1,25(OH)₂D₃ on the adenylate cyclase complex which leads to refractoriness to hormones and forskolin. Our studies of calcitonin binding showed that 1,25(OH)₂D₃ treatment had no effect on calcitonin receptor number or affinity. Although specific binding of the other agonists to their respective receptors was not measured, the fact that refractoriness was induced to a range of stimulating agents made a hormone-receptor mechanism unlikely. It should be pointed out however, that in the case of PTH, half maximally effective hormone concentration was consistently decreased by 1,25(OH)₂D₃ treatment, by at least 10-fold. Thus it is possible that for PTH there is an additional component of the 1,25(OH)₂D₃ effect, perhaps through a decrease in the affinity of PTH for its receptor. This cannot be excluded at this stage because methods to document this are not adequate at present. Forskolin, which has been a useful agent in the study of adenylate cyclase control, is thought to act directly on the catalytic component of adenylate cyclase (Pfeuffer & Metzger, 1982). There is some evidence, however, for a possible role of nucleotide regulatory protein in forskolin action (Daly *et al.*, 1982; Schneyer *et al.*, 1983). The present

observations are similar to those made in a human monocyte line, in which 1,25(OH)₂D₃ treatment induced refractoriness both to forskolin and isoproterenol (Rubin & Catherwood, 1984). Because of the possibility that forskolin might act directly on nucleotide regulatory components as well as catalytic unit of adenylate cyclase, it was necessary to exclude the possibility that the 1,25(OH)₂D₃ effect operated through such a mechanism.

Experiments were therefore carried out to determine whether enhanced N_i unit or diminished N_s unit activities could be responsible for the effect of 1,25(OH)₂D₃. The present results were not consistent with a mechanism operating directly through N_s or N_i. They do not exclude the possibility that 1,25(OH)₂D₃ treatment might result in a lesser activity of a specific subgroup of N_s units, although we think this unlikely since it is not known whether such subgroups exist. Some observations of actions of steroid hormone action indicate possible effects on nucleotide regulatory proteins. For example, in glucocorticoid-deficient animals, coupling of adenylate cyclase was reduced, consistent with a requirement of glucocorticoids for normal N_s unit activity (Davies *et al.*, 1981). On the other hand, treatment of rabbits with luteinizing hormone led to a decrease in corpus luteum N_s activity (Kirchick & Birnbaumer, 1983). At present we conclude, therefore, that the ability of 1,25(OH)₂D₃ to decrease cyclic AMP response to several hormones and forskolin results from an effect of the steroid hormone beyond hormone receptor and nucleotide regulatory proteins, and perhaps directed at the catalytic unit of the adenylate cyclase itself. One possible mechanism for this is that 1,25(OH)₂D₃ alters intracellular availability of Mg²⁺ to the adenylate cyclase complex, thereby altering responses to the hormones and forskolin. This suggestion is made because of the importance of Mg²⁺ in the activation of adenylate cyclase by hormones and forskolin (Martin *et al.*, 1984).

1,25(OH)₂D₃ receptors have been identified in many tissues, and in cells derived from many organs in the body (Eisman, 1983). The possibility that 1,25(OH)₂D₃ is capable of affecting a process as fundamental as that of cyclic AMP generation, and to do so in a sensitive manner, points to important general functions of the vitamin D hormone.

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