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Pretreatment with $1,25(OH)_2$ Vitamin D or $24,25(OH)_2$ Vitamin D Increases Synergistically Responsiveness to Sex Steroids in Skeletal-derived Cells

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We demonstrated previously that vitamin D metabolites modulate the response of bone and cartilage cells to 17β-estradiol (E_2) and dihydrotestosterone (DHT) both in cell cultures and *in vivo*. In the present study, we investigated to what extent pretreatment with $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ would reduce the minimal effective dose of E_2, DHT or progesterone (P) required for stimulation of DNA synthesis and creatine kinase specific activity in cultured osteoblast-like ROS 17/2.8 cells and in rat embryo epiphyseal cartilage cells, and to what extent such pretreatment would increase the maximal response. We measured responses to sex steroids after pretreatment of the cells for 5 days with 0.02% ethanol vehicle or with the vitamin D metabolites $1,25(OH)_2D_3$ (0.12 nM), or $24,25(OH)_2D_3$ (1.2 nM) singly or in combination. Pretreatment of ROS 17/2.8 cells with $1,25(OH)_2D_3$, but not $24,25(OH)_2D_3$, increased synergistically their response to E_2 but not to P, and did not affect their lack of response to DHT. Pretreatment of epiphyseal cartilage cells with either $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ increased synergistically their DNA synthetic response to all three steroids, but their CK response only to E_2 or DHT. The minimal dose for causing a significant response to E_2 in ROS 17/2.8 cells or to either E_2 or DHT in epiphyseal cartilage cells was reduced 10-fold after pretreatment with vitamin D metabolites. After pretreatment, the maximal response was more than doubled in ROS 17/2.8 cells; epiphyseal cartilage cells showed a similar 10-fold decrease in the dose required for maximal response to E_2 or DHT; the improvement in the response to P was significant only for DNA synthesis. We conclude that pretreatment with the appropriate vitamin D metabolite(s) both reduces by an order of magnitude, or more, the amount of sex steroids needed to stimulate skeletal derived cells and increases synergistically the maximal response of the cells.

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INTRODUCTION

Skeletal derived cells, as many others, show cell specific responses to steroid and secosteroid hormones and to peptide hormones. As part of our stepwise approach to the integrated cell type specific action of these different hormones *in vivo*, we demonstrated that bone cells, *in vivo* and *in vitro*, respond in a sex specific manner to gonadal steroids by increased [^{3}H]thymidine incorporation into DNA and increased creatine kinase (CK) specific activity [1]. Cartilage cells from either sex, on the other hand, respond to both androgen and estrogen [1]. In parallel we demonstrated that vitamin D metabolites can stimulate CK and DNA synthesis in skeletal cells, both *in vivo* and *in vitro* [2–5]. These cells, which contain receptors for both gonadal steroids [6–8] and for vitamin D metabolites [2, 5] were therefore highly suitable to study the interactions between gonadal steroids and vitamin D in stimulating their proliferation. Our studies on the combined action of these two classes of hormones revealed that vitamin D metabolites modulated the response to 17β-estradiol (E_2) and to androgens [dihydrotestosterone (DHT), testosterone (T)], both *in vivo* [9] and in cell culture [10, 11] in a cell type specific manner. Bones, but not cartilage, derived from vitamin D-depleted rats, showed a loss of

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response in terms of DNA synthesis and a markedly reduced response to gonadal steroids in terms of CK activity, without changing the sex specificity of the reduced CK response [9]. However, full responsiveness of both parameters was restored by treating the vitamin D-depleted rats with $1,25(OH)_2D_3$ but not $24,25(OH)_2D_3$ [9]. In cell culture, the same cell type specificity and vitamin D metabolite specificity was demonstrated; the response to gonadal steroids was enhanced by $1,25(OH)_2D_3$ in both bone and cartilage cells while $24,25(OH)_2D_3$ enhanced this response only in cartilage cells [11]. This enhancing effect of vitamin D metabolites raises the possibility that lower therapeutic doses of estrogen could be used in cases of post menopausal osteoporosis, which may reduce the threat of estrogen stimulated endometrial cancer and other hazardous side effects, while preserving its protective effect on bone.

In the present study, we investigated this possibility by studying the effect of pretreatment with $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, or both metabolites in combination, on the response of rat osteoblast-like ROS 17/2.8 cells (ROS) [12], and of chondroblast-like rat embryo epiphyseal cartilage cells (Epi) [13] to varying doses of E_2, DHT and progesterone (P), using two markers of bone cell proliferation. We measured increased [^{3}H]thymidine incorporation into DNA and increased specific activity of creatine kinase [2–5] to demonstrate enhanced stimulatory effects of gonadal steroids in these cells. In this and in previous studies of skeletal cells, which contain low concentrations of steroid hormone receptors [6–8, 14], we used the sensitive and rapid post-receptor response marker, the stimulation of CK activity, in addition to stimulation of [^{3}H]thymidine incorporation into DNA. The brain type (BB) isozyme of CK, identified as the major component of the estrogen-induced protein [IP] of rat uterus [15], is part of the "energy buffer" system which regenerates ATP from ADP and thus regulates their cellular concentrations. Modulation of CK activity has been a useful marker for studies on the mechanism of action of hormones [1–4, 9–12, 16–18] because of the

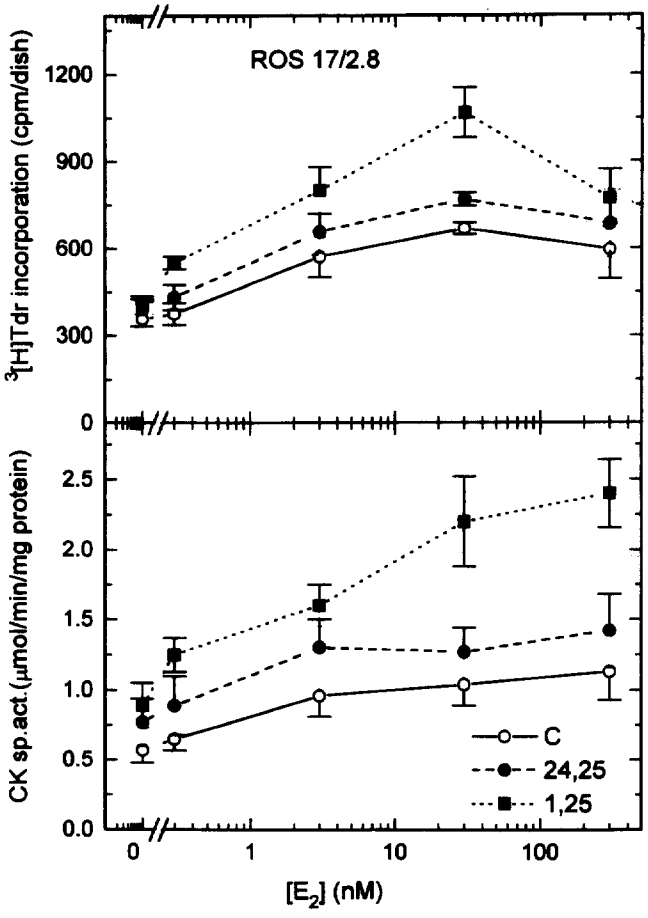

Fig. 1. Stimulation of DNA synthesis and CK specific activity by E_2 in ROS 17/2.8 cells. Cells were pretreated with either vehicle (C, 0.02% ethanol) or 0.12 nM $1,25(OH)_2D_3$ (1,25) or 1.2 nM $24,25(OH)_2D_3$ (24,25) for 5 days. On day 7, cells were challenged with increasing doses of E_2 (0.3–300 nM) for 24 h and then analyzed for DNA synthesis and CK specific activity as described in Materials and Methods. Results are means ± SEM. For DNA synthesis, $n = 5$–15 and for CK, $n = 4$–10. Statistical analysis was made by Student's t-test for experimental vs control values for each pretreatment. In vehicle and $24,25(OH)_2D_3$ pretreated cells, $P \leqslant 0.05$ for 3, 30 and 300 nM E_2, whereas for $1,25(OH)_2D_3$ pretreated cells $P \leqslant 0.05$ for 0.3 and 3 nM E_2 and $P \leqslant 0.01$ for 30 and 300 nM E_2.

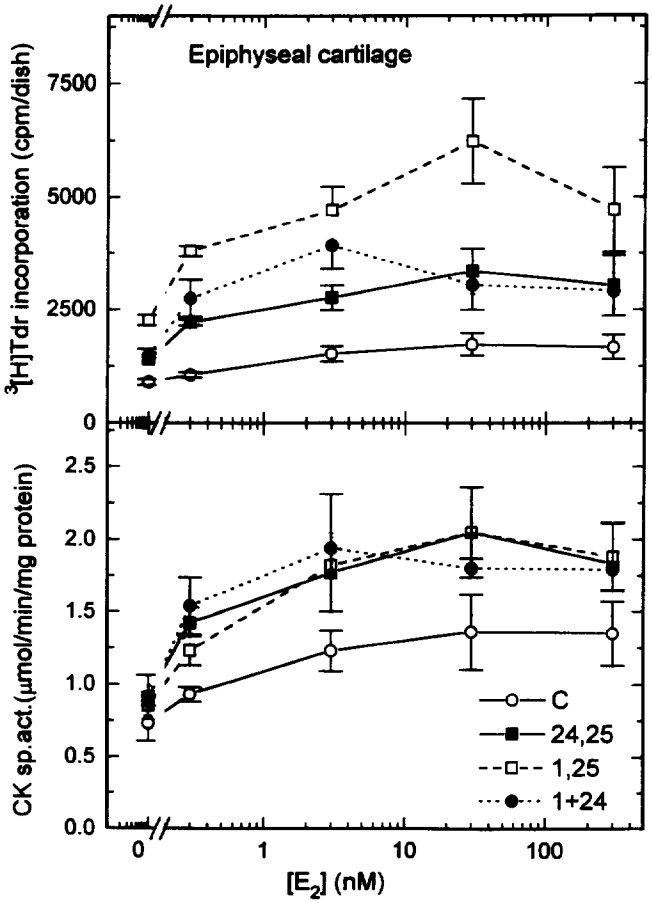

Fig. 2. Stimulation of DNA synthesis and CK specific activity by E_2 in rat epiphyseal cartilage cells. Cells were pretreated with either vehicle (C, 0.02% ethanol) or 0.12 nM $1,25(OH)_2D_3$ (1,25) or 1.2 nM $24,25(OH)_2D_3$ (24,25) or both (1,25 + 24,25) for 5 days. On day 7, cells were challenged with increasing doses of E_2 (0.3–300 nM) for 24 h and then analyzed for DNA synthesis and CK specific activity as described in Materials and Methods. Results are means ± SEM for $n = 5$–15. Statistical analysis was made by Student's t-test for experimental vs control values for each pretreatment. In vehicle pretreated cells, $P \leq 0.05$ for 3, 30 and 300 nM E_2. After pretreatment with $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ or both metabolites, $P \leq 0.05$ for 0.3 and 3 nM E_2 and $P < 0.01$ for 30 and 300 nM E_2.

rapidity and sensitivity of its response. This investigation revealed that, after pretreatment with vitamin D metabolites which increase responsiveness to sex steroids, a 10–100 times lower dose of E_2, DHT or P is capable of stimulating the equivalent proliferative response in the appropriate skeletal derived cells.

MATERIALS AND METHODS

Cell cultures

(1) Osteoblast-like cells, ROS 17/2.8, derived from a rat osteosarcoma [19], were cultured in 35 mm diameter dishes in 2 ml of DMEM + F12 (1 : 1) containing 10% FCS. (2) Rat epiphyseal cell cultures were prepared from rat embryos as described previously [1, 13] and plated in 35 mm diameter dishes in 2 ml BGJ_b medium containing 10% FCS. Their morphological characteristics, alkaline phosphatase activity and content of type II collagen showed these cells to be chondrocytes [13].

Hormonal treatment

Starting 1 day after seeding, the cells were treated with 5 daily additions of either vehicle (C, 0.02% ethanol in saline), or 1.2 nM $1,25(OH)_2D_3$ (0.5 ng/ml), or 12 mM $24,25(OH)_2D_3$ (5 ng/ml), or both metabolites. These doses are an order of magnitude greater than the dissociation constants of the metabolites from the vitamin D receptor. The cells were treated on day 7 after seeding with varying doses of E_2, DHT or P for 24 h and then assayed for CK activity and DNA synthesis.

Creatine kinase assay

Cells were scraped from culture dishes into isotonic homogenization buffer [1] and homogenized using a Polytron homogenizer (Brinkman Instruments, Westbury, N.Y.). Supernatant extracts, obtained by centrifugation at 14,000 *g* for 4 min at 4°C were assayed spectrophotometrically as described previously

[1]. Protein was determined by Coomassie Brilliant Blue dye binding [20] using BSA as standard.

[^{3}H]thymidine incorporation into DNA

After 22 h of hormone treatment, DNA synthesis was measured by pulse labeling with [^{3}H]thymidine for an additional 2 h, as described previously [1]. DNA was determined by Burton's diphenylamine method [21].

Statistical significance

The significance of differences between experimental and control values was evaluated using a non-paired, two-tailed Student's t-test in which n = number of culture dishes.

RESULTS

Since pretreatment with vitamin D metabolites increased the response of skeletal-derived cells to estrogen [10, 11], we performed dose–response studies (Figs 1–4) to determine to what extent pretreatment with $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ could lower the concentration of sex steroids necessary for a defined response and increase the maximal response.

ROS 17/2.8 osteoblast-like osteocarcinoma cells

In the absence of any pretreatment, ROS 17/2.8 cells responded to E_2 by increased DNA synthesis as well as increased CK specific activity in a dose dependent manner, starting at a minimal dose of 3 nM and reaching maximum responses at 30 nM (Fig. 1). DHT was ineffective in stimulating either parameter at any concentration between 3 and 3000 nM; progesterone (P) could stimulate CK specific activity but not DNA synthesis at 6000 nM reaching a maximum response at 60,000 nM (data not shown). Treatment of ROS 17/2.8 cells with either 0.12 nM $1,25(OH)_2D_3$ or with 1.2 nM $24,25(OH)_2D_3$ caused responses which were not as great as those caused by the optimum dose of E_2 (Fig. 1).

Pretreatment of ROS 17/2.8 cells with 0.12 nM $1,25(OH)_2D_3$ (Fig. 1), resulted in synergistic increases in DNA synthesis and CK specific activity throughout the entire range of E_2 doses tested (0.3–300 nM). The minimal effective dose of E_2 for stimulation of both parameters was reduced 10-fold (Fig. 1) and the maximal responses to E_2 of ROS 17/2.8 cells were more than

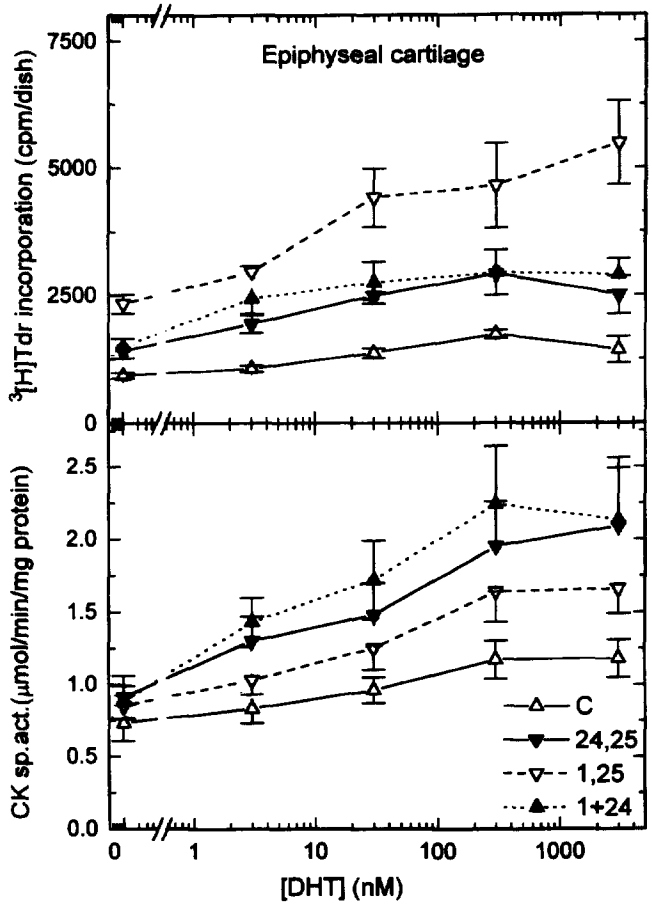

Fig. 3. Stimulation of DNA synthesis and CK specific activity in rat epiphyseal cartilage cells by DHT. Experimental details and statistical analysis are as for Fig. 2, except that DHT was tested at 3–3000 nM. In vehicle pretreated cells, $P \leq 0.05$ for 30, 300 and 3000 nM. After pretreatment with $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ or both metabolites, $P \leq 0.05$ for 3 and 30 nM DHT and $P \leq 0.01$ for 300 and 3000 nM DHT.

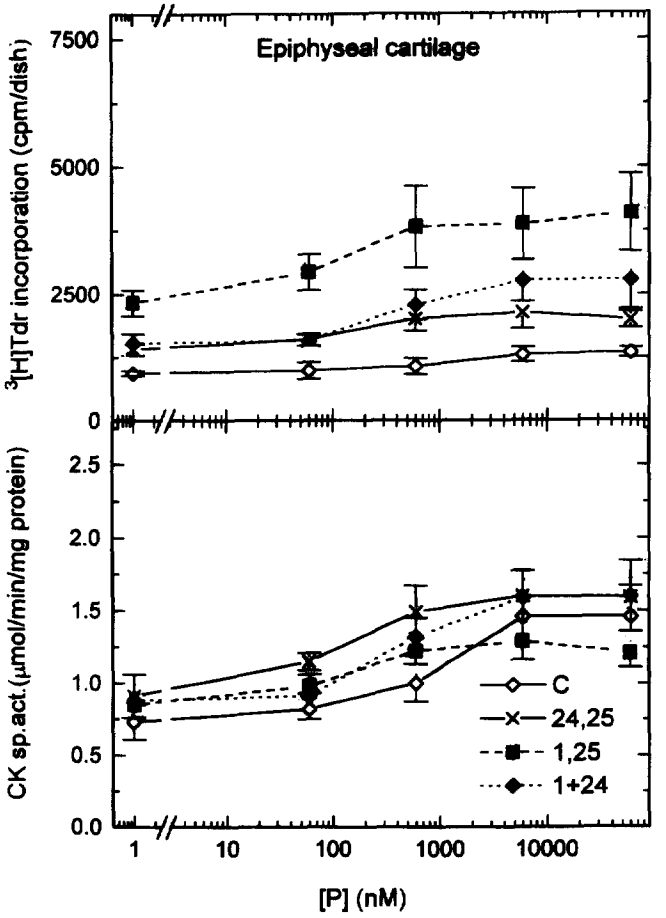

Fig. 4. Stimulation of DNA synthesis and CK specific activity in rat epiphyseal cartilage cells by progesterone. Experimental details and statistical analysis are as for Fig. 2 except that progesterone was used at 60–60,000 nM. In vehicle pretreated cells $P \leqslant 0.05$ for 6000 and 60,000. After pretreatment with either $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ or both metabolites, $P \leqslant 0.05$ for 600 nM and higher concentrations of progesterone for stimulation of [^{3}H]Tdr incorporation, in all cases.

doubled for [^{3}H]thymidine incorporation and more than tripled for CK specific activity.

By contrast, pretreatment with $24,25(OH)_2D_3$ at a concentration 10 times higher than that of $1,25(OH)_2D_3$ caused only a slight increase in stimulation by E_2 which did not reach a value significantly higher than that caused by E_2 alone, at any concentration of E_2 tested. The lack of significant response to DHT was not affected by pretreatment with vitamin D metabolites; the response to P in terms of CK was not augmented, and the lack of stimulation of DNA synthesis by progesterone was not changed by vitamin D pretreatment (data not shown).

Epiphyseal cartilage cells

Untreated epiphyseal cartilage cells responded significantly to minimal doses of 3 nM E_2 (Fig. 2), 30 nM DHT (Fig. 3) as well as to 6000 nM P (Fig. 4) by both increased DNA synthesis and increased CK specific activity, reaching maximum responses at 10-fold higher concentrations of each of these steroids.

Treatment with the vitamin D metabolites either singly or in combination stimulated DNA synthesis in epiphyseal cartilage cells to an equal or greater degree than optimum doses of the sex steroids (Figs 2–4). By contrast, the stimulation of creatine kinase activity by the vitamin D metabolites was less than that caused by moderate doses of the sex steroids (Figs 2–4). Pretreatment with either or both vitamin D metabolites, caused synergistic stimulation by E_2 or DHT at all doses tested and reduced the minimal dose for achieving significant CK or DNA responses to either E_2 or DHT by 10-fold (Figs 2 and 3). The dose of P needed for reaching a significant increase in DNA synthesis was also reduced 10-fold. In the most extreme case, pretreatment with $1,25(OH)_2D_3$ (which caused a 2.4-fold increase in [^{3}H]thymidine incorporation) followed by exposure to 30 nM E_2 (which by itself doubled [^{3}H]thymidine incorporation) resulted in a cumulative stimulation of 7.3-fold (Fig. 2). Comparison of response ratios (Table 1) of cells pretreated with vitamin D metabolites compared with the corresponding control ratio of cells

pretreated with the ethanol vehicle, shows an increase in the ratio in all cases (from 11–63%) for estrogen or androgen pretreated cells. This increase was confined to the DNA synthesis response in the case of P treatment (Fig. 4). Thus, a major factor contributing to the synergistic interactions of vitamin D metabolites and sex steroids appears to be an increase in the responsiveness of epiphyseal cartilage cells caused by the pretreatment with vitamin D metabolites (Table 1).

DISCUSSION

The present results demonstrate that pretreatment with vitamin D metabolites can both increase the maximal acute response to E_2, DHT or P and decrease the dose needed for significant stimulation by increasing the responsiveness of skeletal-derived cells to gonadal steroids. The high doses of P required suggest a non-physiological response, perhaps mediated through the androgen receptors. The sex and cell type specificity of the response [1] was maintained in that ROS 17/2.8 cells responded to E_2 and not DHT while epiphyseal cartilage cells responded to both gonadal steroids [cf. 1,2,9] as was shown *in vivo* [1, 9]. The dosage of $1,25(OH)_2D_3$, appears to be critical, since, at 0.12 nM it both stimulated epiphyseal cartilage cells (Figs 1–4) and augmented the response of epiphyseal cartilage and ROS 17/2.8 cells to gonadal steroids. However, when administered at a 10-fold higher concentration, it caused an inhibition of CK in ROS 17/2.8 cells [12], but had no effect on CK activity in cartilage cells *in vitro* [13] or *in vivo* [2]. On the other hand, $24,25(OH)_2D_3$ was shown to stimulate CK activity in cartilage *in vivo* [2] and *in vitro* [2, 5] when used in at least a 10 times higher concentration than $1,25(OH)_2D_3$ [9].

Although the mechanism by which vitamin D metabolites enhance the action of gonadal steroids on skeletal cells remains to be elucidated, one factor appears to be stimulation of sex hormone receptor synthesis. Western blot analysis using the antiidiotypic antibody against ER (Clone 1D5 was kindly provided by F. Kohen of the Department of Hormone Research, The Weizmann Institute of Science [22]) revealed an increase in ER in epiphyseal cartilage cells after $24,25(OH)_2D_3$ pretreatment and an even greater increase after $1,25(OH)_2D_3$ pretreatment [23]. The same increase in ER by $1,25(OH)_2D_3$ was also seen [23] in rat embryo calvaria cells [9, 10] as well as by immunoassay in $SaOS_2$ cells [Fournier and Sömjen, to be published]. Previous studies on the interaction between estrogens and $1,25(OH)_2D_3$ demonstrated that $1,25(OH)_2D_3$ increases the concentration of E_2 receptors in human bone explants [24, 25]. It was also shown that 96 h exposure to 10^{-8} M $1,25(OH)_2D_3$ caused a 2–4-fold increase in the number of estrogen receptors in bone marrow-derived stromal cells and in MC3T3 cells [26]. It is possible that there is also a reciprocal stimulation of vitamin D receptors by estrogen, as has been found in the rat uterus [27].

The low concentration of E_2 receptors and androgen receptors [24] in cultured bone cells [6–8], the finding of E_2 receptors in bone cells obtained from a patient [14] with polyostotic fibrous dysplasia (McCune-Albright syndrome), and the failure to identify E_2 receptors in bone cells from osteoporotic women [14] suggest that the concentration of hormone receptors of bone cells is an important factor in determining their biological response to E_2 or DHT. Vitamin D metabolites appear to be part of the hormonal milieu that stimulates the expression of E_2 receptors in bone and cartilage cells. The increase in the E_2 receptor concentration caused by vitamin D metabolites in rat epiphyseal cartilage cells and female derived calvaria cells could, in turn, raise the responsiveness of cells to E_2. A parallel stimulation of androgen receptors is an additional possibility. Such an increase could be significant, in light of the low concentrations of E_2 and probably androgen receptors in skeletal cells [6–8, 24]. It is possible, therefore, that in skeletal cells (and perhaps in other responsive tissues) the increase in estrogen (and probably androgen) receptors is part of the stimulating action of vitamin D metabolites leading to an augmented response to gonadal steroids.

Table 1. Increase in maximal responsiveness of epiphyseal cartilage cells to sex steroids after pretreatment with vitamin D metabolites

		Response to combined treatment / Response to pretreatment alone	
Pretreatment	Treatment	CK	DNA
Vehicle (0.02% EtOH)	E_2 (30)	1.9	2.0
$1,25(OH)_2D_3$	E_2 (30)	2.5	2.7
$24,25(OH)_2D_3$	E_2 (30)	2.2	2.4
$1,25(OH)_2D_3 + 24,25(OH)_2D_3$	E_2 (3)	2.2	2.6
Vehicle (0.02% EtOH)	DHT (300)	1.6	1.8
$1,25(OH)_2D_3$	DHT (300)	2.0	2.0
$24,25(OH)_2D_3$	DHT (300)	2.1	2.0
$1,25(OH)_2D_3 + 24,25(OH)_2D_3$	DHT (300)	2.6	2.2

In all cases there was a significant difference between vitamin D pretreated and vehicle preteated cells, but no significance among the vitamin D metabolite pretreated cells.

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