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RECIPROCAL MODULATION BY SEX STEROID AND CALCIOTROPHIC HORMONES OF SKELETAL CELL PROLIFERATION

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Summary—We have demonstrated previously that 17β-estradiol (E_2) stimulates cell proliferation in skeletal tissues, as measured by increased DNA synthesis and creatine kinase (CK) specific activity, and that calciotrophic hormones modulate E_2 activity in rat osteoblastic sarcoma cells (ROS 17/2.8). Moreover, E_2 failed to stimulate DNA synthesis in vitamin D-depleted female rat bone in the absence of prior i.p. injections of $1,25(OH)_2D_3$. We have, therefore, studied the effects of pretreatment of cells by one hormone on their response to challenge by a second hormone. We now report reciprocal interactions of sex steroids and other hormones modulating bone formation on cell proliferation parameters in primary bone and cartilage cell cultures; these interactions can selectively augment or diminish cell responsiveness to a given hormone.

Pretreatment of rat epiphyseal cartilage cell cultures with $1,25(OH)_2D_3$, $24,25(OH)_2D_3$ or parathyroid hormone (PTH) for 5 days, followed by E_2 treatment for 24 h, resulted in increased DNA synthesis compared to cultures pretreated with vehicle. Prostaglandin (PGE_2) pretreatment blocked further response to E_2. In the reciprocal case, rat epiphyseal cartilage cells, pretreated with E_2, showed an increased response to PTH, a loss of the response to PGE_2 or $24,25(OH)_2D_3$ and an inhibition of CK activity and DNA synthesis by $1,25(OH)_2D_3$, similar to the characteristic inhibitory action of $1,25(OH)_2D_3$ in osteoblasts. By contrast, rat epiphyseal cartilage cells pretreated with testosterone showed no changes in response to PTH, $24,25(OH)_2D_3$ or PGE_2 and a decreased response to E_2, but were stimulated by $1,25(OH)_2D_3$.

Rat embryo calvaria cell cultures behaved similarly to epiphyseal cartilage cultures except that $24,25(OH)_2D_3$ pretreatment did not increase the response to E_2. Reciprocally, pretreatment with E_2 before exposure to calciotrophic hormones did not change the responses of rat embryo calvaria cell cultures to $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$.

These findings suggest that the mutual interactions between calciotrophic hormones and E_2, demonstrated here *in vitro*, could selectively affect the responses of bone and cartilage cells to E_2 by several mechanisms. These possibilities include increased E_2 receptors and E_2-stimulated differentiation of cartilage cells to more E_2 responsive cells showing some characteristics of osteoblasts.

INTRODUCTION

It is well established by clinical and laboratory studies that estrogens strongly influence bone metabolism, and that estrogen deficiency is a principal cause of postmenopausal osteoporosis [1–4]. Although the results of several studies suggested that estrogen acts indirectly [5–8], recent papers have described direct actions of E_2 on bone [9–11] and cartilage cells [12] and identified specific estrogen receptors in bone derived cells [13–15]. Androgens, as well, were found to stimulate bone cell proliferation *in vitro* [16]. We have found that estrogen can influence proliferation (DNA synthesis) and energy metabolism (creatine kinase activity) in several osteoblastic models, including ROS 17/2.8 cells, rat bone and cartilage derived cells in culture, and in diaphysis and epiphysis of female rats [17]. Furthermore, we have demonstrated that vitamin D metabolites can modulate the response to E_2 both *in vivo* and *in vitro* [18]. Moreover, we have shown that pretreatment of ROS 17/2.8 cells with $1,25(OH)_2D_3$ augmented the stimulation by E_2, while no effect of E_2 was seen after pretreatment with PGE_2. Reciprocally, E_2 pretreatment caused the PGE_2 response to decline below control levels while showing insignificant effects on responses to the other hormones tested [19].

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In these studies, in addition to determining [^{3}H]thymidine incorporation, a classical marker of cell proliferation, we utilized the stimulation of the brain type (BB) isozyme of creatine kinase (CK, EC 2.7.3.2), as a rapid and convenient early enzymic marker which precedes cell division. CK, involved in the "energy buffer" system which regulates the cellular concentrations of ATP and ADP, was identified as the estrogen induced protein (IP) of the rat uterus [20]. CK activity therefore became a useful marker for studies of estrogen action in organs containing E_2 receptors [21]. We have broadened the use of this marker and demonstrated that calciotrophic hormones such as PGE_2, parathyroid hormone (PTH) and vitamin D metabolites increase CK activity, within an hour or two of administration, in skeletal derived cells *in vivo* and *in vitro* [22, 23]. Thus, CK activity is a convenient response marker for hormones, which, like estrogen, increase cell growth [21–25]. Since bone and cartilage derived cells contain receptors for vitamin D secosteroids [26], PTH [27, 28] and PGE_2 [27, 28], as well as for gonadal steroids [13–15], all of which have a trophic action on these cells, we studied, in the present work, the reciprocal relationship between gonadal steroids and other calciotrophic hormones during the process of induction of cell proliferation in skeletal derived cell cultures. This study utilized non-transformed cells since previous data are available only on the rat osteosarcoma cell line ROS 17/2.8 [19].

EXPERIMENTAL

Two experimental model systems were used. Rat epiphyseal cartilage cells [29] grown in BGJ_b medium supplemented with 10% fetal calf serum, were derived from both female and male rats, since no sex specificity was shown in cartilage [17]. Rat embryo calvaria cell cultures [23] were grown in BGJ_b medium lacking Ca^{2+}, supplemented with 10% fetal calf serum. The cells were grown in low Ca^{2+} medium in order to enrich the culture with osteoblasts [23]. Cell cultures were treated with 17β-estradiol (E_2, 30 nM) after 5 daily additions of either vehicle (0.02% ethanol in saline), $1,25(OH)_2D_3$ (0.5 ng/ml), $24,25(OH)_2D_3$ (5 ng/ml), PTH (10 nM), PGE_2 (500 ng/ml) or the combinations $1,25(OH)_2D_3 + 24,25(OH)_2D_3$ or PTH + PGE_2. Conversely, cultures were pretreated with E_2 (3 nM) or testosterone (T, 30 nM) for 5 days and then with either PTH (10 nM), PGE_2 (500 ng/ml), E_2 (30 nM), T (300 nM), $1,25(OH)_2D_3$ (0.5 ng/ml) or $24,25(OH)_2D_3$ (5 ng/ml). The doses of the hormones chosen were the maximal effective doses found in previous experiments [22, 23]. Either creatine kinase activity, [^{3}H]thymidine incorporation into DNA, or both these markers of cell proliferation were determined, 24 h after the last addition of either vehicle or hormones to subconfluent cultures.

CK preparation and assay

Cells were scraped from culture plates and homogenized by sonication in homogenization buffer containing isotonic sucrose [17]. Supernatant extracts were obtained by centrifugation of homogenates at 12,000 ***g*** for 5 min at 4°C in an Eppendorf microcentrifuge. CK activity was measured in a Gilford 250 automatic recording spectrophotometer at 340 nm using a coupled assay described previously [17]. A unit of enzyme activity was defined as the amount yielding 1 μmol ATP/min at 30°C, and specific activity as μmol/min/mg protein. Protein was determined by Coomassie brilliant blue dye binding [30] using bovine serum albumin as the standard.

[^{3}H]thymidine incorporation into DNA

Cells were treated as for the CK assay, for 22 h, and then were incubated for an additonal 2 h with [^{3}H]thymidine (5 μCi/ml, 5 Ci/mmol, Amersham, Bucks, England) and analysed as described previously [17] utilizing solubilization in 0.3 to 1.0 N NaOH of the final acid insoluble pellet. DNA was determined by the method of Burton [31].

Reagents

Vitamin D metabolites were a gift from Professor Samuel Edelstein, Department of Biochemistry, The Weizmann Institute of Science. Bovine PTH(1–84) (trichloroacetic acid powder) and other biochemicals were obtained from the Sigma Chemical Co., St Louis, Mo. Chemical reagents used were of analytical grade.

Statistical significance

The significance of differences between experimental and control values was evaluated using Student's t-test in which n = number of culture dishes. The experiments were repeated 2–4 times.

RESULTS

Since in our previous work [18] we found that rat epiphyseal cartilage cell cultures and rat embryo calvaria cell cultures showed different patterns of response to vitamin D metabolites and to E_2 after pretreatment with vitamin D metabolites, we employed, in the present study, both types of non-transformed cell cultures, namely epiphyseal cartilage cultures, consisting of a high proportion of chondroblasts and osteoblast-enriched cultures of embryo calvaria cells. We used 5 days pretreatment, since in preliminary experiments (unpublished), only after 3 days or more of pretreatment was there a significant change in responsiveness.

Rat epiphyseal cartilage cell cultures: modulation by calciotrophic hormone pretreatment of the response to E_2

Using DNA synthesis as a direct measure of cell proliferation (Fig. 1) we found a greater than 2-fold augmentation of the rate of estrogen stimulated [^{3}H]thymidine incorporation after $1,25(OH)_2D_3$ pretreatment of epiphyseal cartilage cell cultures. the metabolite, $24,25(OH)_2D_3$ which by itself stimulated [^{3}H]thymidine incorporation more than 2-fold, caused an even greater augmentation of the response to E_2 (Fig. 1). We reported recently [17] the parallel result that pretreatment of rat epiphyseal cartilage cell cultures for 5 days with $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ increase by 2.5-fold the stimulation of creatine kinase activity by E_2.

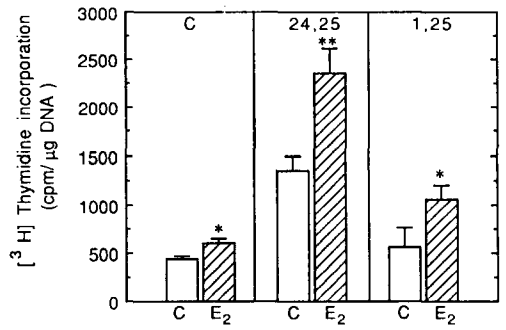

Fig. 1. Increased stimulation of DNA synthesis by E_2 after pretreatment of rat epiphyseal cartilage cell cultures with vitamin D metabolites. Cells were pretreated daily, from day 2 through 6 after seeding, with either control vehicle (C; 0.02% ethanol in saline) or $24,25(OH)_2D_3$ (24,25; 5 ng/ml) or $1,25(OH)_2D_3$ (1,25; 0.5 ng/ml). Cultures in each group were then treated for 24 h with vehicle or with 30 nM E_2. DNA synthesis was assayed as described in Materials and Methods. Results are means ± SE for $n = 8$, (n is the total number of culture dishes from ⩾2 independent experiments). $*P \leqslant 0.05$, $**P \leqslant 0.01$ by Student's t-test for C vs E_2 values. The control means for $24,25(OH)_2D_3$ pretreated cells is greater than the untreated control mean ($P \leqslant 0.01$).

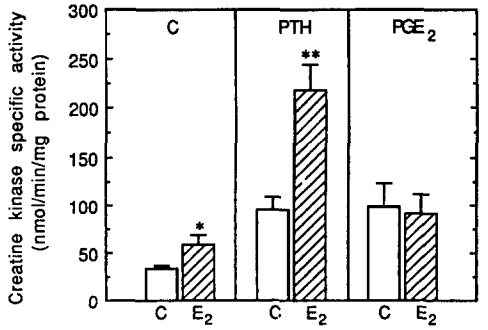

Fig. 2. Modulation of the stimulation of CK activity by E_2 after pretreatment of rat epiphyseal cartilage cell cultures with calciotrophic hormones. Cells were pretreated daily, from day 2 to 6 after seeding, with either vehicle or PTH (10 nM) or PGE_2 (1.5 µM). Cultures in each group were then treated for 24 h with vehicle or with 30 nM E_2. CK activity was assayed as described in Materials and Methods. Results are means ± SE for $n = 8$. $*P \leqslant 0.05$, $**P \leqslant 0.01$ by Student's t-test for C vs E_2 values. The control means for PTH or PGE_2 pretreated cells are greater than the untreated control mean ($P \leqslant 0.01$).

We have shown that rat osteosarcoma (ROS 17/2.8) cells are more responsive to E_2 after PTH pretreatment [19]. When rat epiphyseal cartilage cell cultures were pretreated daily with PTH, there was a significant increase in the response to E_2 (Fig. 2) as measured by CK activity. However, after comparable pretreatment with PGE_2 there was no additional stimulation by E_2. In these cartilage cells, either PTH or PGE_2 pretreatment, in themselves, stimulated CK activity more than 2-fold.

Rat epiphyseal cartilage cell cultures: modulation by E_2 pretreatment of the response to other hormones

Since we have shown above that calciotrophic hormones modify the response of the cells to E_2, we wished to study the reciprocal interaction, namely, the effect of pretreatment with E_2 before exposure to other hormones. Pretreatment of rat epiphyseal cartilage cell cultures for 5 days with E_2 increased the CK response to PTH (Fig. 3) but did not modify either the lack of stimulation of CK activity by T or the stimulation by a 10-fold higher dose of E_2. However, E_2 pretreatment abolished the response to PGE_2 and to $24,25(OH)_2D_3$. This is unlike the effect on osteoblasts—either ROS 17/2.8 cells [19] or primary rat embryo calvaria cell cultures (see below). E_2 pretreatment also caused significant inhibition of CK activity by $1,25(OH)_2D_3$ in contrast to the lack of response to $1,25(OH)_2D_3$ after control pretreatment. Parallel results were seen when changes in DNA synthesis were monitored (Fig. 4).

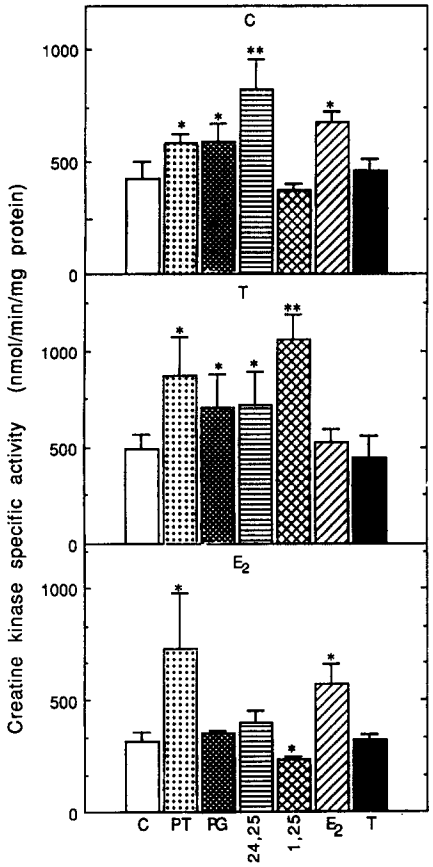

Fig. 3. Modulation of the CK response to calciotropic and sex steroid hormones after pretreatment of rat epiphyseal cartilage cell cultures with E_2 or testosterone (T). Cells were pretreated daily from day 2 to 6 after seeding, with either vehicle (C), E_2 (3 nM) or T (30 nM). Cultures in each group were then treated for 24 h with vehicle, PTH (PT; 10 nM); PGE_2 (PG; 500 ng/ml); $24,25(OH)_2D_3$ (24,25; 5 ng/ml); $1,25(OH)_2D_3$ (1,25; 0.5 ng/ml); E_2 (30 nM) or T (300 nM). CK activity was assayed as described in Materials and Methods. Results are means ± SE for $n = 9$. $*P \leq 0.05$, $**P \leq 0.01$ by Student's t-test for experimental vs control values for the appropriate pretreatment. In this experiment, the control means for untreated and T or E_2 pretreated cells do not differ significantly.

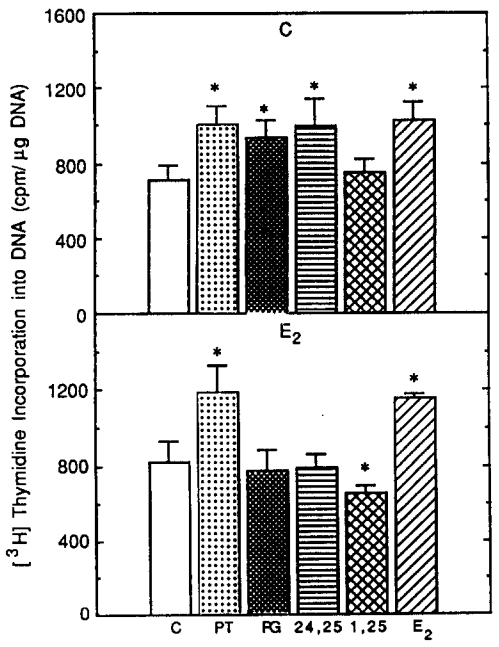

Fig. 4. Modulation of the proliferative response to calciotrophic and sex steroid hormones after pretreatment of rat epiphyseal cartilage cell cultures with E_2. The abbreviations and experimental details are the same as in the legend to Fig. 3, except that DNA synthesis was assayed instead of CK activity. In this experiment, the control means do not differ significantly.

Rat epiphyseal cartilage cell cultures: modulation by T pretreatment of the response to other hormones

In order to determine whether the effects of E_2 shown above on the response of epiphyseal cartilage cells to calciotrophic hormones are sex steroid specific, we compared 5 days pretreatment with vehicle to pretreatment for 5 days with T (Fig. 3). T pretreatment did not significantly change the extent of the stimulation of CK by PTH, or PGE_2. However, T pretreatment decreased significantly the response to $24,25(OH)_2D_3$, and at the same time caused a stimulatory response to $1,25(OH)_2D_3$.

The stimulation of CK activity by E_2 was prevented by T pretreatment (Fig. 3). However, in contrast to the additional stimulation found when a 10-fold higher E_2 concentration was tested after E_2 pretreatment, a 10-fold higher concentration of T did not provoke any rise in CK activity after T pretreatment.

When DNA synthesis was measured (Fig. 5), comparable results were obtained with some quantitative changes: while the E_2 response appeared to be lowered by T pretreatment, [^{3}H]thymidine incorporation nevertheless remained significantly higher than in vehicle pretreated control cultures.

Rat embryo calvaria cells: modulation by hormone pretreatment of the response to E_2

Rat embryo calvaria cell cultures responded to pretreatment with $1,25(OH)_2D_3$ with more than a doubling in their response to E_2 stimulation (Fig. 6) similar to rat epiphyseal cartilage cell cultures (Fig. 1). While $24,25(OH)_2D_3$ also stimulated CK activity by itself to the same extent as did $1,25(OH)_2D_3$, the increased activity after pretreatment with $24,25(OH)_2D_3$ was slightly less (close to twice the control value). Pretreatment with the combination of both vitamin D metabolites followed by E_2 resulted in a CK specific activity not significantly greater

than that after pretreatment with $1,25(OH)_2D_3$ alone (Fig. 6).

Rat embryo calvaria cell cultures pretreated with PTH (Fig. 6), showed a doubling in their CK activity response to E_2 but there was no augmented response to E_2 after pretreatment with PGE_2 alone or with the combination of PGE_2 + PTH (Fig. 6). Although pretreatment with the combination of PGE_2 + PTH by itself caused an additive stimulation of CK specific activity, it prevented the further increase by E_2 seen after pretreatment with PTH alone.

Rat embryo calvaria cell cultures: lack of modulation by E_2 pretreatment of the response to vitamin D metabolites

In reciprocal experiments, rat embryo calvaria cell cultures pretreated with E_2 and then treated for an additional 24 h with either $24,25(OH)_2D_3$ or $1,25(OH)_2D_3$ or E_2, showed no change in their response pattern (Fig. 7) as measured by [^{3}H]thymidine incorporation, in contrast to the modified responses shown by

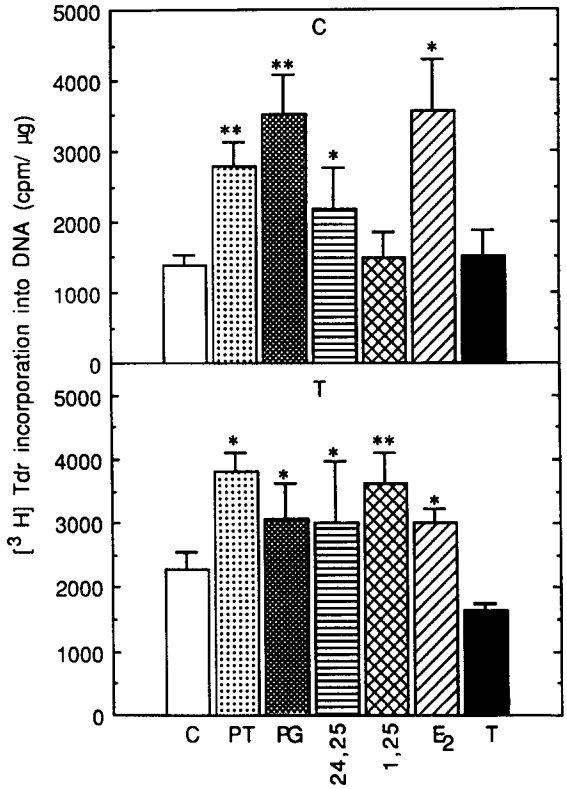

Fig. 5. Modulation of the proliferative response to calciotrophic and sex steroid hormones after pretreatment of rat epiphyseal cartilage cell cultures with T (30 nM). The abbreviations and experimental details are the same as in the legend to Fig. 3 except that DNA synthesis was assayed instead of CK activity. In this experiment, the control mean for T pretreated cells is greater than the control mean for untreated cells ($P \leq 0.05$).

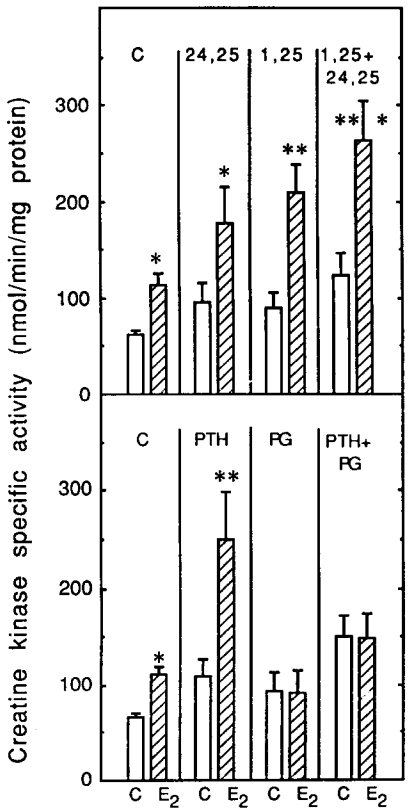

Fig. 6. Modulation of the stimulation of CK activity by E_2 after pretreatment of rat embryo calvaria cell cultures with calciotrophic hormones. Cells were pretreated daily, from day 2 to 6 after seeding, with either vehicle (C), $24,25(OH)_2D_3$ (24,25; 5 ng/ml; $1,25(OH)_2D_3$ (1,25; 0.5 ng/ml); both 1,25 plus 24,25; PTH (10 nM); prostaglandin E_2 (PG; 500 ng/ml) or both PTH plus PGE_2. Cultures in each group were then treated for 24 h with vehicle or with 30 nM E_2. CK activity was assayed as described in Materials and Methods. Results are means ± SE for $n = 6$–12. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.005$ by Student's t-test for experimental vs control values. The control means for single pretreatments with $24,25(OH)_2D_3$, $1,25(OH)_2D_3$ or PTH are significantly greater than the control mean ($P \leq 0.05$). Control means for combined pretreatments by $24,25(OH)_2D_3 + 1,25(OH)_2D_3$ or PTH + PGE_2 are greater (highly significant, $P \leq 0.01$) than the untreated control mean. For greater ease of visual comparison the untreated control mean ($n = 12$) is presented in both the upper and lower panel.

epiphyseal cartilage cell cultures (Fig. 3). We previously found that in ROS 17/2.8 cells, another osteoblast model, E_2 pretreatment did not modify the response to any hormone except to PGE_2 [19].

Although the calvaria cell cultures were exposed for 5 days to a stimulatory dose of E_2 they were still able to show a further positive response to a single 10-fold higher dose of E_2.

DISCUSSION

We have broadened and extended our previous observations [18, 19] that vitamin D

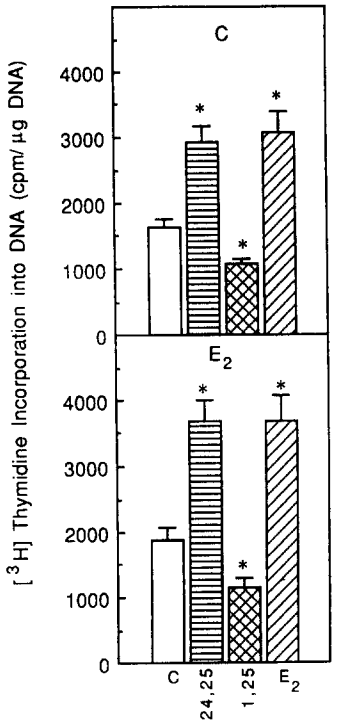

Fig. 7. Modulation of the stimulation of DNA synthesis by vitamin D metabolites after pretreatment of rat embryo calvaria cell cultures with E_2. Cells were pretreated daily, from day 2 to 6 after seeding, with either vehicle (C) or E_2 (3 nM). Cultures in each group were then treated for 24 h with vehicle (C) or $24,25(OH)_2D_3$ (24,25; 5 ng/ml); $1,25(OH)_2D_3$ (1,25; 0.5 ng/ml) or a 10-fold higher concentration of E_2 (30 nM). DNA synthesis was assayed as described in Materials and Methods. Results are means ± SE for $n = 9$. $*P \leq 0.05$, by Student's t-test for experimental vs control values for the appropriate pretreatment. In this experiment the control means do not differ significantly.

metabolites, particularly $1,25(OH)_2D_3$ increase the responses of skeletal-derived cells to E_2 and we now show modulations of steroid hormone action by other calciotrophic hormones such as PTH and PGE_2. Additionally, we have found in untransformed primary cell cultures a reciprocal modulation by E_2 of response to calciotrophic hormones, as previously reported for ROS 17/2.8 osteoblast-like cell cultures [19].

As found in the ROS 17/2.8 cell model [19], in both rat epiphyseal cartilage cell cultures (Figs 1 and 2) and osteoblast-like rat embryo calvaria cell cultures (Fig. 6), $1,25(OH)_2D_3$ or PTH increased the responsiveness to estrogen, whereas PGE_2 prevented the proliferative response to E_2. The cell specificity of the mitotic response was shown by the observation that the response to E_2 of rat epiphyseal cartilage cell cultures increased after pretreatment with $24,25(OH)_2D_3$, while there was no augmented response to E_2 in rat embryo calvaria cell cultures, or after pretreatment with $24,25(OH)_2D_3$, in the osteoblast-like ROS 17/2.8 cells [19], as shown previously.

In the reciprocal interaction, pretreatment of rat embryo calvaria cell cultures with E_2 did not change responses to vitamin D metabolites (Fig. 7). However, in rat epiphyseal cartilage cell cultures, E_2 pretreatment increased the response to PTH, and prevented the response to either PG or $24,25(OH)_2D_3$ (Figs 3 and 4). Since E_2 pretreatment also led to significant inhibition of CK activity by $1,25(OH)_2D_3$ (compared to no significant response to $1,25(OH)_2D_3$ with vehicle pretreatment), it is possible that E_2 pretreatment favors differentiation and/or maturation of the epiphyseal cartilage cells to cells showing some of the characteristics of osteoblasts (as do ROS 17/2.8 cells). Such characteristics include a growth inhibitory response to $1,25(OH)_2D_3$, growth stimulation by PTH and E_2, and a lack of response to PGE_2 or $24,25(OH)_2D_3$ [19, 32, 33]. This differentiation effect appears to be specific to E_2, since T affected the cartilage cells differently (c.f. Figs 4 and 5), for example by increasing the stimulation by $1,25(OH)_2D_3$. Measurement of other markers specific for bone and cartilage will be necessary to test this hypothesis of differentiation.

Although pretreatment of both cell types with E_2 for 5 days still permitted them to respond to a 10-fold higher concentration of E_2 (Figs 3, 4 and 7; Ref. [19]), pretreatment with E_2 did not change the lack of response to T. On the other hand, T pretreatment of cartilage cells, or ROS 17/2.8 cells [19] led to loss of the CK response of the cells to E_2; nor was there any stimulation by a 10-fold higher dose of T itself. Since E_2 and T act physiologically at different concentrations, we plan to obtain complete dose–response curves to compare directly E_2 and T effects for both transformed and primary cells.

Possible mechanisms for reciprocal hormonal interactions

The interactions of the different types of hormones in modulation of bone and cartilage cell proliferation described in this study and previously [19] may involve a variety of mechanisms at different biological levels. These mechanisms, based on established actions of the hormones involved, include, but are not limited to:

1. Regulation of genes for protooncogenes, growth factors, receptors and hormone synthesiz-

ing' enzymes. The induction of mRNA for CK [34], *c-fos* [35] and other early proteins associated with cell division is stimulated by both steroid and peptide hormones and by PGE_2 in bone derived cells. Estrogenic activation of the UMR106 osteoblastic cell line was proposed to occur via stimulation of IGF-I formation [36] which is also modulated by $1,25(OH)_2D_3$. A particularly important protein which can be induced by one hormone is the receptor for another. For example, $1,25(OH)_2D_3$ has recently been reported to increase the concentration of E_2 receptors in human bone explants [37]. The reciprocal increase in $1,25(OH)_2D_3$ receptors by E_2 treatment in rat uterus [38] had been reported previously. In humans, while E_2 receptors were not detected in bone cells of osteoporotic women [39], they were found, in bone cells from a patient with polyostotic fibrous dysplasia (McCune-Albright Syndrome) [39]. Additionally, an enzyme required for synthesis of another hormone can be stimulated in postmenopausal women; estrogen increases the circulating levels of $1,25(OH)_2D_3$ by increasing the activity of $1,25(OH)_2D_3$ hydroxylase [40], leading to increased calcium absorption [41].

II. Control of differentiation and proliferation. A 5-day pretreatment was used in the present study to ensure sufficient time for differentiation or selective proliferation of particular cell types. Indeed, we found that while E_2 did not affect the response to vitamin D metabolites or to itself in differentiated osteoblast-like cells, it may have caused maturation of cartilage cells to cells which respond like osteoblasts, as judged by the characteristic loss of response to $24,25(OH)_2D_3$ and PGE_2 and the acquisition of response to $1,25(OH)_2D_3$ [19, 32, 33]. Alternatively and/or additionally, a hormone such as E_2 may cause differentiation of cells which preferentially respond to other hormones.

III. Regulation of second messengers. In addition to its interaction with vitamin D metabolites, E_2 also interacts with the PTH and PGE_2 systems (Figs 2–6). PTH increased responsiveness to E_2 in both cartilage and calvaria cells. Reciprocally, E_2 increased the proliferative response to PTH of epiphyseal cartilage cells but not calvaria cells or osteoblast-like ROS 17/2.8 osteosarcoma cells [19]. Although there was no modification of the proliferative responses of osteoblast cultures to PTH, E_2 has been found [42], to modulate selectively the ability of hPTH to enhance the production of cAMP in human osteoblast-like cells (but not in ROS 17/2.8 cells), by inhibiting adenylate cyclase stimulation [42], either via the PTH receptor or receptor specific G-protein coupling and transduction. E_2 was also found to enhance renal responsiveness to exogenous PTH administration in elderly female subjects [43]. In addition, estrogen replacement therapy was recently found to influence PTH function in postmenopausal women by increasing the sensitivity of PTH stimulation by calcium [44].

IV. Antagonism between hormones. The fact that E_2 and PGE_2 antagonize each other's proliferative action (Figs 3 and 4) is a provocative observation. If several days exposure to E_2 indeed caused cartilage cells to respond like osteoblasts, it would explain why, as in ROS 17/2.8 osteoblast-like cells [19], E_2 pretreated cartilage cells did not respond to PGE_2 [32, 33]. Thus, if part of estrogen's mechanism of action is stimulation of bone formation via inhibition of PGE_2 production [7], it would be intriguing to determine, for example, how addition of PGE_2 prevents maximal E_2 action after PTH pretreatment (Fig. 6).

At present, there is little data to support any detailed mechanism for the interactions observed in this study and further experiments are needed to understand the specific mechanisms involved. However, regardless of how E_2 and calciotrophic hormones modulate each others actions on rat bone and cartilage cells, if human bone responds similarly, there may be clinical implications in the findings reported here, particularly during puberty and in the peri- and postmenopausal period. Although estrogen replacement therapy is at present the preferred preventive treatment for postmenopausal osteoporosis [45], recent findings [46] have renewed the discussion [47] of the contribution of treatment with estrogen (and estrogen combined with progesterone) to the incidence of breast cancer. Therefore, it is important to determine, for example, if raising concentrations of calciotrophic hormones to the optimum, or lowering PGE_2 concentrations to the minimum, or both, would permit reduction of the estrogen dose required for safe and effective prevention or therapy of osteoporosis in women.

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Forschung, GmBH, Braunschweig, F.R.G. A.M.K. is the Joseph Moss Professor of Molecular Endocrinology.

REFERENCES

1. Riggs B. L. and Melton III L. J.: Involutional osteoporosis. *New Engl. J. Med.* **314** (1986) 1676–1686.
2. Raisz L. G.: Local and systemic factors in the pathogenesis of osteoporosis. *New Engl. J. Med.* **318** (1988) 818–828.
3. Riis B. J., Rodbro P. and Christiansen C.: The role of serum concentrations of sex steroids and bone turnover in the development and occurrence of post-menopausal osteoporosis. *Calc. Tiss. Int.* **38** (1986) 318–322.
4. Ivey J. L. and Baylink J. D.: Postmenopausal osteoporosis. Proposed roles of defective coupling and estrogen deficiency. *Metab. Bone. Dis. Relat. Res.* **3** (1981) 3–7.
5. Caputo C. B., Meadows D. and Raisz L. G.: Failure of estrogens and androgens to inhibit bone resorption in tissue culture. *Endocrinology* **98** (1976) 1065–1068.
6. Gallagher J. C., Riggs R. L. and DeLuca H. F.: The effect of estrogen on calcium absorption and serum vitamin D metabolites in post menopausal osteoporosis. *J. Clin. Endocr. Metab.* **51** (1980) 1359–1364.
7. Feyen J. H. M. and Raisz L. G.: Prostaglandin production by calvaria from sham operated and oophorectomized rats: effect of 17β-estradiol *in vivo*. *Endocrinology* **121** (1987) 819–821.
8. Pansini F., Bergamini C. M., Bellinazzi A., Andreoli M., Perri G., Bagni B. and Mollica G.: Ovarian steroids modulate the action of calcitonin in women. *J. Endocr.* **116** (1988) 155–159.
9. Gray T. K., Flynn T. C., Gray K. M. and Nabell L. M.: 17β-estradiol acts directly on the clonal osteoblast cell line UMR106. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987) 6267–6271.
10. Ernst M., Schmid Ch. and Froesch E. R.: Enhanced osteoblast proliferation and collagen gene expression by estradiol. *Proc. Natn. Acad. Sci. U.S.A.* **85** (1988) 2307–2310.
11. Goulding A. and Gold E.: The effects of chronic prednisolone treatment on bone resorption and bone composition in intact and ovariectomized rats and in ovariectomized rats receiving 17β-estradiol. *Endocrinology* **122** (1988) 482–487.
12. Corvol M. T., Carracosa A., Tsagris L., Blanchard O. and Rappaport R.: Evidence for a direct *in vitro* action of sex steroids on rabbit cartilage cells during skeletal growth: influence of age and sex. *Endocrinology* **120** (1987) 1422–1429.
13. Komm B. S., Terpening C. M., Benz D. J., Graeme K. A., Gallegos A., Korc M., Greene G. L., O'Malley B. W. and Hausler M. R.: Estrogen binding, receptor mRNA, and biologic response in osteoblast-like osteosarcoma cells. *Science* **241** (1988) 81–84.
14. Eriksen E. F., Colvard D. S., Berg N. J., Graham M. L., Mann K. G., Spelsberg T. C. and Riggs B. L.: Evidence of estrogen receptors in normal human osteoblast-like cells. *Science* **241** (1988) 84–86.
15. Dayani M., Corvol M. T., Robel P., Eychenne B., Moncharmont B., Tsafris L. and Rappaport R.: Estrogen receptors in cultured rabbit articular chondrocytes. Influence of age. *J. Steroid Biochem.* **31** (1988) 351–356.
16. Kasperk C. H., Wergedal J. E., Farley J. R., Linkhart T. A., Turner R. T. and Baylink D. J.: Androgen directly stimulates proliferation of bone cells *in vitro*. *Endocrinology* **124** (1989) 1576–1578.
17. Sömjen D., Weisman Y., Harell A., Berger E. and Kaye A. M.: Direct and sex specific stimulation by sex steroids of creatine kinase activity and DNA synthesis in rat bone. *Proc. Natn. Acad. Sci. U.S.A.* **86** (1989) 3361–3365.
18. Sömjen D., Kaye A. M., Harell A. and Weisman Y.: Modulation by vitamin D status of the responsiveness of rat bone to gonadal steroids. *Endocrinology* **125** (1989) 1870–1876.
19. Kaye A. M., Weisman Y., Harell A. and Sömjen D.: Hormonal stimulation of bone cell proliferation. *J. Steroid Biochem. Molec. Biol.* **37** (1990) 431–435.
20. Reiss N. and Kaye A. M.: Identification of the major component of the estrogen induced protein of rat uterus as the BB isozyme of creatine kinase. *J. Biol. Chem.* **256** (1981) 5741–5749.
21. Malnick S. D. H., Shaer A., Soreq H. and Kaye A. M.: Estrogen induced creatine kinase in the reproductive system of the immature female rat. *Endocrinology* **113** (1983) 1907–1909.
22. Sömjen D., Weisman Y., Binderman I. and Kaye A. M.: Stimulation of creatine kinase BB activity by 1α,25-dihydrocholecalciferol and 24R,25-dihydrocholecalciferol in rat tissues. *Biochem. J.* **219** (1984) 1037–1041.
23. Sömjen D., Kaye A. M. and Binderman I.: Stimulation of creatine kinase BB activity by parathyroid hormone and by prostaglandin E_2 in cultured bone cells. *Biochem. J.* **225** (1985) 591–596.
24. Sömjen D., Kaye A. M. and Binderman I. 24R,25-dihydroxy vitamin D stimulates creatine kinase BB activity in chick cartilage cells in culture. *FEBS Lett.* **167** (1984) 281–284.
25. Sömjen D., Binderman I. and Weisman Y.: The effects of 24R,25-dihydrocholecalciferol and 1,25-dihydrocholecalciferol on ornithine decarboxylase activity and on DNA synthesis in the epiphysis and diaphysis of rat bone and in the duodenum. *Biochem. J.* **214** (1983) 293–298.
26. Sömjen D., Earon Y., Harell S., Shimshoni Z., Weisman Y., Harell A., Kaye A. M. and Binderman I.: Developmental changes in responsiveness to vitamin D metabolites. *J. Steroid Biochem.* **27** (1987) 807–813.
27. Peck W. A., Burks J. K., Wilkin J., Rodan S. B. and Rodan G. A.: Evidence for preferential effects of parathyroid hormone, calcitonin and adenosine on bone and periosteum. *Endocrinology* **100** (1977) 1357–1364.
28. Wong G. L., Kent G. N., Ku K. Y. and Cohn D. V.: The interaction of parathormone and calcium on the hormone-regulated synthesis of hyaluronic acid and citrate decarboxylation in isolated bone cells. *Endocrinology* **103** (1978) 2274–2282.
29. Levy J., Shimshoni Z., Sömjen D., Berger E., Fine N., Silberman M. and Binderman I.: Rat epiphyseal cells in culture: responsiveness to bone seeking hormones. *In vitro* **24** (1988) 620–625.
30. Bradford M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72** (1976) 248–254.
31. Burton K.: A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62** (1956) 315–323.
32. Sömjen D., Kaye A. M., Rodan G. A. and Binderman I.: Regulation of creatine kinase activity in rat osteogenic sarcoma cell clones by parathyroid hormone, prostaglandin E_2 and vitamin D metabolites. *Calc. Tiss. Int.* **37** (1985) 635–638.
33. Majeska R. J. and Rodan G. A.: The effect of $1,25(OH)_2D_3$ on alkaline phosphatase in osteoblastic osteosarcoma cells. *J. Biol. Chem.* **257** (1982) 3362–3365.
34. Sömjen D., Weisman Y., Harell A., Binderman I. and Kaye A. M.: Accumulation of mRNA for creatine kinase BB is stimulated by calcium regulating hormones. *J. Endocr. Invest.* **11** (Suppl. 1) (1988) 22 (Abstr.).

35. Sömjen D., Berger E., Weisman A., Binderman I. and Kaye A. M.: Accumulation of mRNA for the proto-oncogene c-fos is stimulated by calcium regulating hormones. *Ann. Meet. Israel Endocrine Soc.*, Jerusalem (1989) p. 82 (Abstr.).
36. Gray T. K., Mohan S., Linkhart T. A. and Baylink D. J.: Estradiol stimulates *in vitro* the secretion of insulin-like growth factors by the clonal osteoblastic cell line, UMR106. *Biochem. Biophys. Res. Commun.* **158** (1989) 407–412.
37. Arnett T. R. and Colston K. W.: Actions of 1,25-dihydroxy vitamin D and estradiol in human osteosarcoma cell lines. *J. Bone Miner. Res.* **3** (1988) S80 (Abstr.).
38. Walters M. R.: An estrogen stimulated 1,25-dihydroxy vitamin D receptor in rat uterus. *Biochem. Biophys. Res. Commun.* **103** (1981) 721–726.
39. Kaplan D. S., Fallon M. D., Boden S. D., Schmidt R., Senior M. and Haddad G. J.: Estrogen receptors in bone in a patient with polyostotic fibrous dysplasia (McCune-Albright Syndrome) *New Engl. J. Med.* **319** (1988) 421–425.
40. Cheema C., Grant B. F. and Marcus R.: Effect of estrogen on circulating "free" and total 1,25-dihydroxy vitamin D and on the parathyroid vitamin D axis in postmenopausal women. *J. Clin. Invest.* **83** (1989) 537–542.
41. Gallagher J. C., Riggs R. L. and DeLuca H. F.: Effect of estrogen on calcium absorption and serum vitamin D metabolites in postmenopausal osteoporosis. *J. Clin. Endocr. Metab.* **51** (1980) 1359–1364.
42. Fukayama S. and Tashjian A. H.: Direct modulation by estradiol of the response of human bone cells (SaOS-2) to human parathyroid hormone (PTH) and PTH related protein. *Endocrinology* **124** (1989) 397–401.
43. Imanaka S., Morimoto S., Onishi T., Takamoto S., Fukyo K., Koh E., Hoh K., Hironaka T., Shiraishi T., Morita R., Taniguchi K. and Kamahara Y.: Effect of estrogens on renal responsiveness to parathyroid hormone in elderly female subjects. *Endocr. Jap.* **35** (1988) 593–599.
44. Boucher A., D'Amour P., Hamel L., Fugére P., Gasone-Barre M., Lepage R. and Ste-Moril L. G.: Estrogen replacement decreases the set point of parathyroid hormone stimulation by calcium in normal postmenopausal women. *J. Clin. Endocr. Metab.* **68** (1989) 831–836.
45. Lindsay R.: Sex steroids in the pathogenesis and prevention of osteoporosis. In *Osteoporosis: Etiology, Diagnosis and Management* (Edited by B. L. Riggs and L. J. Melton III). Raven Press, New York (1988) pp. 333–358.
46. Bergkvist L., Adami H.-O., Persson I., Hoover R. and Schairer C.: The risk of breast cancer after estrogen and estrogen-progestin replacement. *New Engl. J. Med.* **321** (1989) 293–297.
47. Barrett-Connor E.: Postmenopausal estrogen replacement and breast cancer. *New Engl. J. Med.* **321** (1989) 319–320.