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The Effects of 17β-Estradiol on Chondrocyte Differentiation Are Modulated by Vitamin D_3 Metabolites

Z. Schwartz,[1,3,4] Y. Finer,[4] E. Nasatzky,[4] W. A. Soskolne,[4] D. D. Dean,[1] B. D. Boyan,[1,2,3] and A. Ornoy[5]

Departments of [1]Orthopaedics, [2]Biochemistry, and [3]Periodontics, The University of Texas Health Science Center at San Antonio, TX; and Departments of [4]Periodontics, Faculty of Dental Medicine, and [5]Anatomy and Embryology, Faculty of Medicine, Hebrew University Hadassah, Jerusalem, Israel

Both 17β-estradiol (17β) and the vitamin D metabolites, 1,25-$(OH)_2D_3$(1,25) and 24,25-$(OH)_2D_3$ (24,25), regulate endochondral bone formation in vivo and in vitro. The effects of 17β are sex-specific and cell maturation-dependent. Similarly, the effects of 1,25 and 24,25 are cell maturation-dependent, with 1,25 affecting growth zone chondrocytes (GC) and 24,25 affecting resting zone chondrocytes (RC). This study examined whether the response of chondrocytes to 17β is altered after pretreatment with 1,25 or 24,25. Cells were isolated from the costochondral cartilage of male or female rats. Confluent, fourth-passage GC and RC cultures were pretreated with 1,25 or 24,25, respectively, for 24 or 48 h followed by treatment with 17β for an additional 24 h. At harvest, cell proliferation ([^{3}H]-thymidine incorporation), differentiation (alkaline phosphatase specific activity [ALPase]), general metabolism ([^{3}H]-uridine incorporation), and proteoglycan production ([^{35}S]-sulfate incorporation) were determined. 1,25 enhanced the inhibitory effect of 17β on [^{3}H]-thymidine incorporation by female GC cells; in contrast, no effect was observed in GC cells obtained from male rats. When male RC cells were treated with 17β, [^{3}H]-thymidine incorporation was inhibited; however, when these cells were pretreated with 24,25 for 48 h, 17β stimulated [^{3}H]-thymidine incorporation 24,25 had no effect on 17β-dependent [^{3}H]-thymidine incorporation by female RC cells. 17β stimulated ALPase in female GC cells, but had no effect on male GC cells. 1,25 pretreatment of female GC cells inhibited the stimulatory effect of 17β on ALPase, but had no effect on ALPase in male GC cultures. 17β had no effect on male RC cell ALPase and stimulated ALPase in female RC cells. This was not affected by pretreatment with 24,25. Pretreatment with 1,25 increased the basal level of sulfate incorporation only in female GC. No effect was found in RC cells. These results indicate that pretreatment of rat costochondral chondrocytes with vitamin D metabolites modulate the effect of 17β. Although the effect of vitamin D metabolites alone on these chondrocytes is maturation-dependent and not sex-specific, the influence of preincubation with vitamin D metabolites on the effect of 17β is hormone-specific, sex-specific, and maturation-dependent.

Key Words: Chondrocyte cultures; 1,25-$(OH)_2D_3$; 24,25-$(OH)_2D_3$; 17β-estradiol; alkaline phosphatase; thymidine; proteoglycan.

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Author to whom all correspondence and reprint requests should be addressed: Barbara D. Boyan, PhD, Department of Orthopaedics, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284-7774. E-mail: MESSIER@uthscsa.edu

Introduction

It is well established that estrogens play a major role in the regulation of mammalian bone growth and development, and that many estrogen effects are mediated indirectly by other hormones and local factors secreted by cells in response to estrogen stimulation *(1–8)*. Recently, however, a large body of data accumulated from both in vivo *(9,10)* and in vitro *(11–15)* studies strongly suggest that estrogens exert direct effects on cells as well. In cartilage, the direct effects of 17β-estradiol (17β) include inhibition of chondrocyte proliferation *(11–15)* and stimulation of total protein production measured by RNA synthesis *(15,16)*, matrix production of both proteoglycan *(15,17,18)*, and collagen *(15,19)*, as well as cell differentiation measured by the specific activity of alkaline phosphatase *(15,16)*, phospholipase A_2 *(20)*, and creatine kinase *(21)*. In addition, many of these direct effects on chondrocytes are gender-specific *(14–19,22–27)*, and some of them are cell maturation-dependent *(15,19)*. It has been traditionally accepted that the direct effects of estrogens are mediated through classic steroid hormone receptor mechanisms, and specific receptors for estrogens have been found in articular cartilage *(28–32)*, growth plate *(33)*, fracture callus *(34)*, and costochondral chondrocytes *(22)*. However, recent evidence also suggests that some of the direct effects of 17β are mediated through nongenomic mechanisms *(20)*.

Some of the response of mesenchymal cells to 17β appears to be modulated by vitamin D metabolites. 17β failed to stimulate DNA synthesis in bone of vitamin D-depleted female rats, whereas the effect of this sex hormone was restored after adding vitamin D_3 *(27)*. Furthermore, pretreatment of rat epiphyseal cell cultures *(35)* with 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$, or ROS 17/2.8 osteoblast-like cells *(36)* with 1,25-$(OH)_2D_3$, enhanced the effects of 17β. Moreover, 1,25-$(OH)_2D_3$ treatment of bone marrow-derived stromal cells that express functional estrogen receptors caused a twofold increase in the concentration of the estrogen receptor *(37)*.

The two vitamin D metabolites, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, play active roles in cartilage differentiation and maturation during endochondral bone formation *(38,39)*. Previous studies indicate that cartilage cells have 24,25-$(OH)_2D_3$ receptors *(40–44)*, as well as 1,25-$(OH)_2D_3$ receptors *(45,46)*. It was demonstrated that chondrocytes at distinct stages of endochondral maturation respond differentially to these two vitamin D metabolites *(47)*. The cellular responses include changes in cell proliferation *(48)*, alkaline phosphatase *(47,49)*, and phospholipase A_2 specific activities *(49)*, extracellular matrix protein synthesis *(48)*, calcium flux *(50)*, protein kinase C activity *(51)*, and prostaglandin E_2 production *(52)*. Taken together, the studies indicate that 1,25-$(OH)_2D_3$ primarily targets growth zone cartilage cells, the more mature chondrocytes, and 24,25-$(OH)_2D_3$ targets the less mature resting zone cartilage cells.

Accordingly, the aim of the present study was to examine whether the response of growth zone chondrocytes (GC) and resting zone chondrocytes (RC) to 17β is modulated by pretreatment with 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$, respectively, and to find out whether such modulation is sex-specific or cell maturation-dependent. We used a chondrocyte culture system in which chondrocytes at two different stages of maturation are isolated from two different zones of costochondral cartilage from male or female rats *(47,48,53)*. These studies were not designed to assess the vitamin D metabolite specificity of the cellular response, since we did not treat growth zone cells with 24,25-$(OH)_2D_3$, nor were resting zone cells exposed to 1,25-$(OH)_2D_3$.

Results

[^{3}H]-Thymidine Incorporation

Exposure to 17β for 24 h caused a dose-dependent inhibition of [^{3}H]-thymidine incorporation by female GC chondrocytes. Inhibition was significant at 10^{-8}–10^{-7} *M* (Figs. 1A and 2A). Pretreatment with 10^{-9} or 10^{-8} *M* 1,25-$(OH)_2D_3$ for 24 h did not change this effect (Fig. 1B,C). However, pretreatment with 1,25-$(OH)_2D_3$ for 48 h enhanced the inhibitory effect of 17β on [^{3}H]-thymidine incorporation in female GC cells (Fig. 2B,C), which was significant at 10^{-10}–10^{-6} *M*. 17β had no effect on male GC chondrocytes whether or not the cells were pretreated with 1,25-$(OH)_2D_3$ for 24 (Fig. 1) or 48 (Fig. 2) h.

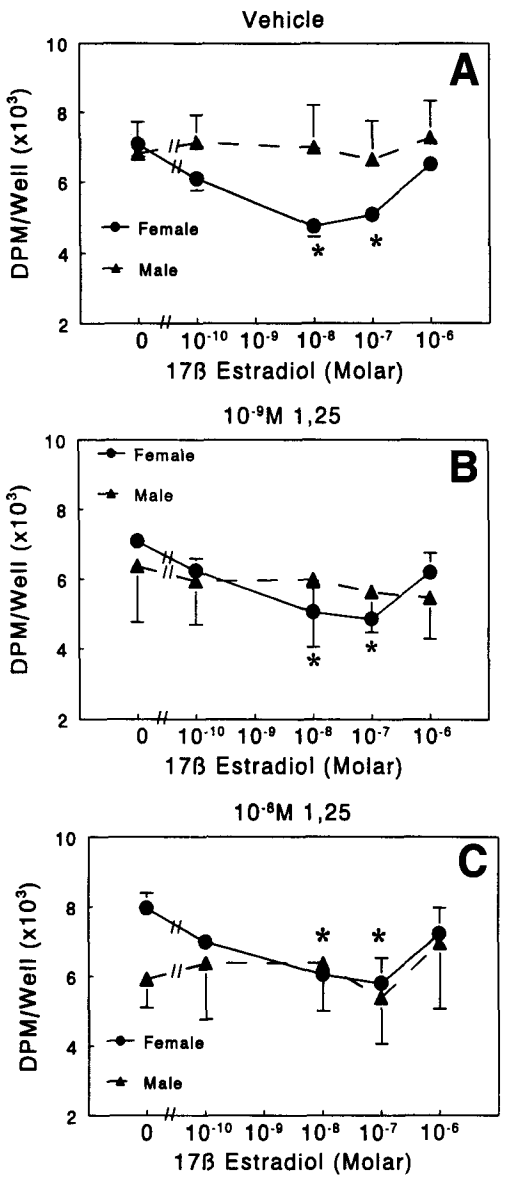

Fig. 1. Effect of pretreatment with 1,25-$(OH)_2D_3$ for 24 h on [^{3}H]-thymidine incorporation by GC in response to 17β. Confluent, fourth-passage cultures of GC from male and female rats were pretreated with control vehicle (**A**), 10^{-9} *M* 1,25-$(OH)_2D_3$ (**B**), or 10^{-8} *M* 1,25-$(OH)_2D_3$ (**C**) for 24 h. At the end of pretreatment, the media were replaced with fresh media containing 10^{-10}–10^{-6} *M* 17β, the incubation continued for another 24 h, and [^{3}H]-thymidine incorporation by the cells was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments. *$P < 0.05$ for treatment vs control.

17β inhibited [^{3}H]-thymidine incorporation by female RC cells that had been pretreated for 24 h with control media (Fig. 3A). The effect was significant at 10^{-10}–10^{-7} *M* 17β. When the cells were pretreated with 24,25-$(OH)_2D_3$ for 24 h, no change in response to 17β was noted (Fig. 3B,C). 17β also inhibited [^{3}H]-thymidine incorporation by female chondrocytes pretreated with control media for 48 h (Fig. 4A). Pretreatment with 10^{-8} *M* (Fig. 4B) or 10^{-7} *M* (Fig. 4B) 24,25-$(OH)_2D_3$ did not alter this response.

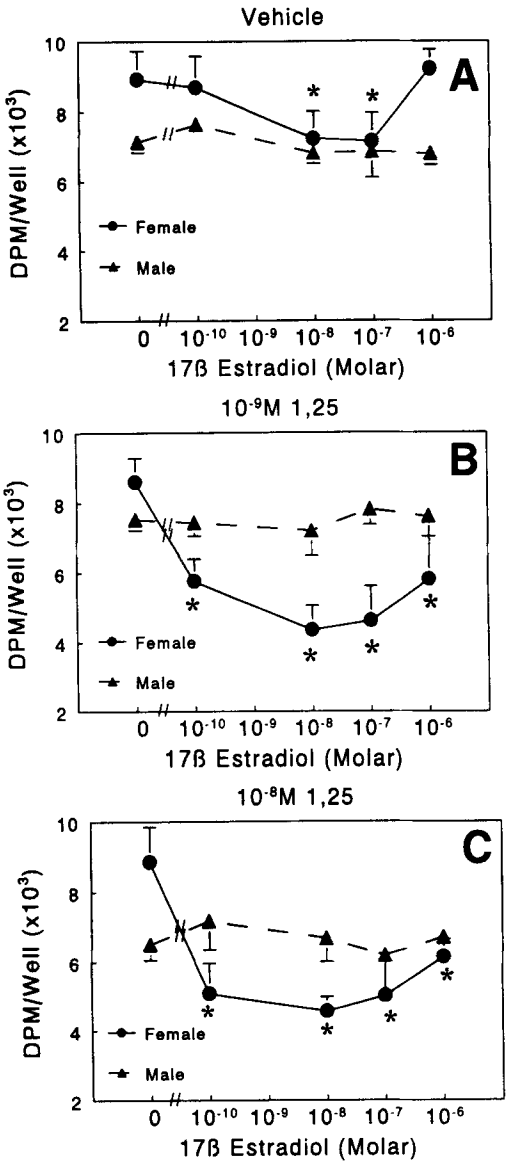

Fig. 2. Effect of pretreatment with 1,25-$(OH)_2D_3$ for 48 h on [^{3}H]-thymidine incorporation by GC in response to 17β. Confluent, fourth-passage cultures of GC from male and female rats were pretreated with control vehicle (**A**), 10^{-9} *M* 1,25-$(OH)_2D_3$ (**B**), or 10^{-8} *M* 1,25-$(OH)_2D_3$ (**C**) for 48 h. At the end of pretreatment, the media were replaced with fresh media containing 10^{-10} to 10^{-6} *M* 17β, the incubation continued for another 24 h, and [^{3}H]-thymidine incorporation by the cells was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments. *$P < 0.05$ for treatment vs control.

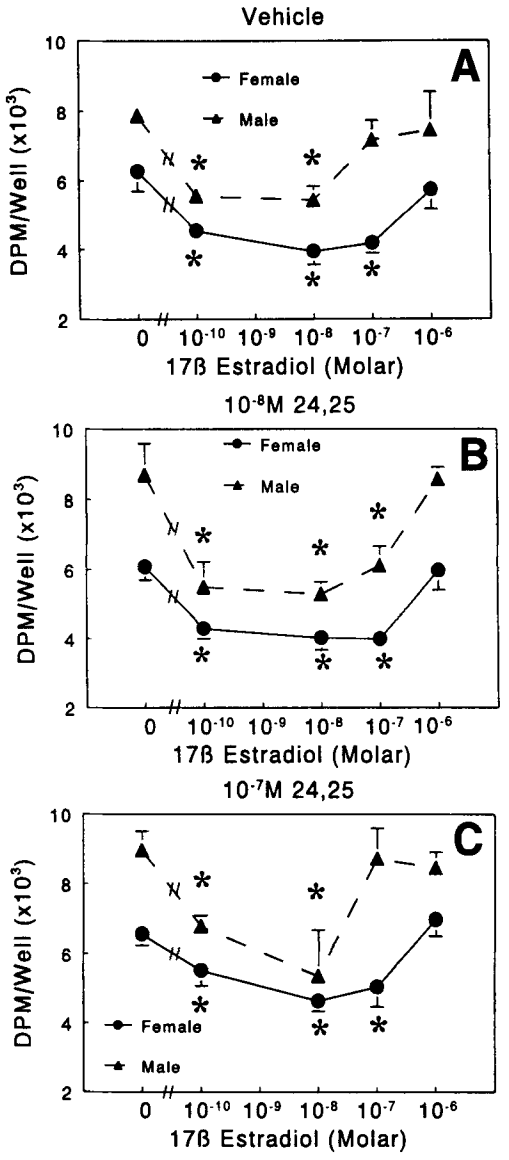

Fig. 3. Effect of pretreatment with 24,25-$(OH)_2D_3$ for 24 h on [^{3}H]-thymidine incorporation by RC in response to 17β. Confluent, fourth-passage cultures of RC from male and female rats were pretreated with control vehicle (**A**), 10^{-8} *M* 24,25-$(OH)_2D_3$ (**B**), or 10^{-7} *M* 24,25-$(OH)_2D_3$ (**C**) for 24 h. At the end of pretreatment, the media were replaced with fresh media containing 10^{-10}–10^{-6} *M* 17β, the incubation continued for another 24 h, and [^{3}H]-thymidine incorporation by the cells was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments. *$P < 0.05$ for treatment vs control.

Similarly, 17β inhibited [^{3}H]-thymidine incorporation by male RC cells pretreated with control media for 24 h (Fig. 3A). The inhibition was significant at 10^{-10}–10^{-8} *M* hormone (Fig. 3A). Pretreatment with 10^{-8} *M* 24,25-$(OH)_2D_3$ for 24 h did not alter cellular response to 17β, although the cells treated with 10^{-7} *M* 24,25-$(OH)_2D_3$ became sensitive to 10^{-7} *M* 17β (Fig. 3B). Pretreatment with 10^{-8} or 10^{-7} *M* 24,25-$(OH)_2D_3$ for 48 h reversed the effect of 17β on [^{3}H]-thymidine incorporation from inhibition to stimulation, significant at 10^{-8}–10^{-7} *M* 17β (Fig. 4B,C).

Alkaline Phosphatase Specific Activity (ALPase)

17β caused an increase in ALPase in cultures of GC chondrocytes from female rats pretreated with control media for 24 (data not shown) or 48 (Fig. 5A) h. The stimulation was biphasic and significant at 10^{-8}–10^{-7} *M*. Pretreatment with 10^{-9} or 10^{-8} *M* 1,25-$(OH)_2D_3$ for 24 (data not

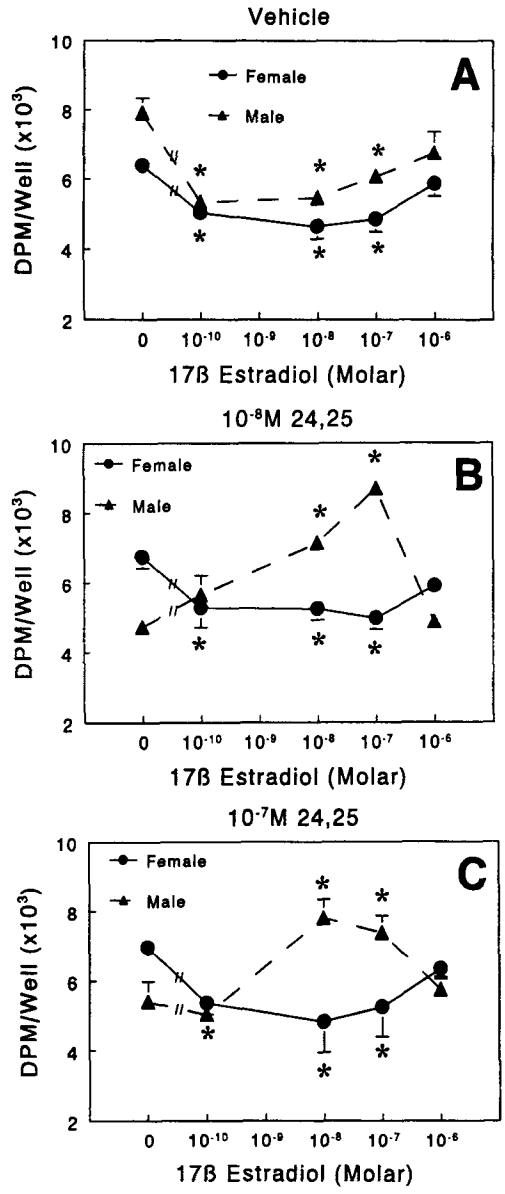

Fig. 4. Effect of pretreatment with 24,25-$(OH)_2D_3$ for 48 h on [^{3}H]-thymidine incorporation by RC in response to 17β. Confluent, fourth-passage cultures of RC from male and female rats were pretreated with control vehicle (**A**), 10^{-8} *M* 24,25-$(OH)_2D_3$ (**B**), or 10^{-7} *M* 24,25-$(OH)_2D_3$ (**C**) for 48 h. At the end of pretreatment, the media were replaced with fresh media containing 10^{-10}–10^{-6} *M* 17β, the incubation continued for another 24 h, and [^{3}H]-thymidine incorporation by the cells was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments. *$P < 0.05$, treatment vs control.

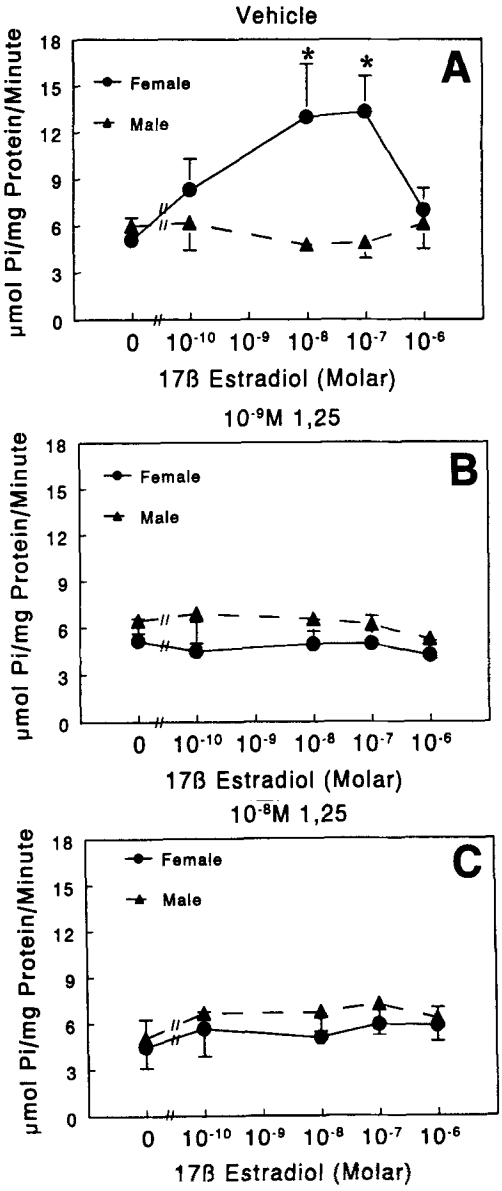

Fig. 5. Effect of pretreatment with 1,25-$(OH)_2D_3$ for 48 h on ALPase of GC in response to 17β. Confluent, fourth-passage cultures of GC from male and female rats were pretreated with control vehicle (**A**), 10^{-9} *M* 1,25-$(OH)_2D_3$ (**B**), or 10^{-8} *M* 1,25-$(OH)_2D_3$ (**C**) for 48 h. At the end of pretreatment, the media were replaced with fresh media containing 10^{-10}–10^{-6} *M* 17β, the incubation continued for another 24 h, and ALPase in the cell layer was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments. *$P < 0.05$, treatment vs control.

shown) or 48 h (Fig. 5B,C) abolished the biphasic stimulatory effect of the hormone. Comparison of the data from five independent experiments confirmed the observations described above. As shown in Fig. 6, pretreatment with 1,25-$(OH)_2D_3$ for 48 h inhibited the stimulatory effect of 10^{-8} *M* 17β on ALPase. The effect of 1,25-$(OH)_2D_3$ was dose-dependent and, at 10^{-7} *M*, had totally abolished response to 17β.

17β had no effect on alkaline phosphatase in male GC cells, whether the cultures were preincubated in control media for 24 (data not shown) or 48 (Fig. 5A) h. Pretreatment with 1,25-$(OH)_2D_3$ did not alter this response, whether the cells were incubated for 24 (data not shown) or 48 (Fig. 5B,C) h with the vitamin D metabolite.

17β increased alkaline phosphatase in female RC cells pretreated with control media for 24 (data not shown) or 48 (Fig. 7A) h. Effects of the hormone were significant at 10^{-8}–10^{-7} *M*. The response to 17β was not altered by pretreatment of the cultures with 24,25-$(OH)_2D_3$ for either 24

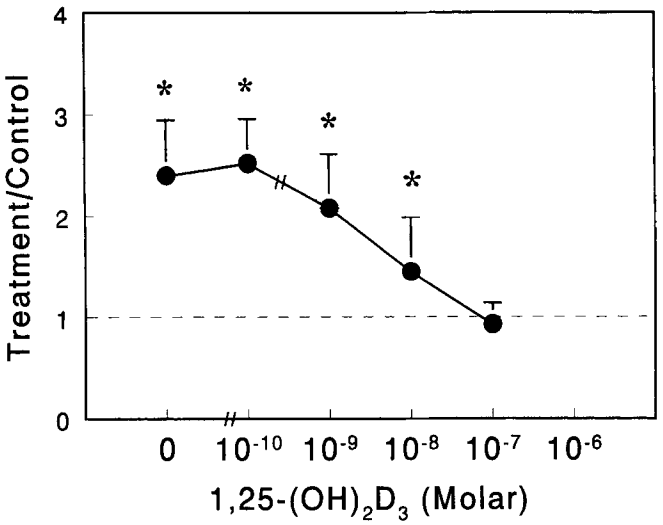

Fig. 6. Treatment/control ratios for ALPase of female growth zone cell cultures pretreated with 1,25-$(OH)_2D_3$, followed by treatment with 10^{-8} *M* 17β. Confluent, fourth-passage GC from female rats were preincubated with 10^{-10}–10^{-6} *M* 1,25-$(OH)_2D_3$ for 48 h, followed by treatment with 10^{-8} *M* 17β for 24 h. At harvest, ALPase in the cell layer was measured. Values are the mean ± SEM of five experiments. $^*P < 0.05$, treatment/control vs 1.0.

(data not shown) or 48 (Fig. 7B,C) h. 17β had no effect on male RC cells, nor was this altered by exposure to 24,25-$(OH)_2D_3$.

[^{3}H]-Uridine Incorporation

17β caused a significant increase in [^{3}H]-uridine incorporation in female GC cells pretreated in control media for 24 (Fig. 8A) or 48 (data not shown) h. Pretreatment with 1,25-$(OH)_2D_3$ did not alter this response. No effect on [^{3}H]-uridine incorporation was observed when 17β was added to male GC cells (Fig. 8A), and pretreatment with 10^{-9} or 10^{-8} *M* 1,25-$(OH)_2D_3$ for 24 h (Fig. 8B,C) or 48 h (data not shown) did not change that.

In female RC cells, 17β caused a biphasic inhibition of [^{3}H]-uridine incorporation in cultures pretreated with control media for 24 (Fig. 9A) or 48 (data not shown) h. The inhibition was significant at 10^{-10}–10^{-7} *M*. In cultures pretreated with 10^{-8} or 10^{-7} *M* 24,25-$(OH)_2D_3$ for 24 h, the inhibitory effect of 10^{-10} *M* 17β was abolished, but 10^{-7} and 10^{-8} *M* 17β continued to suppress [^{3}H]-uridine incorporation (Fig. 9B,C).

Male RC cells exhibited a biphasic inhibition of [^{3}H]-uridine incorporation in response to 10^{-8}–10^{-7} *M* 17β (Fig. 9A). Pretreatment with 10^{-8} *M* 24,25-$(OH)_2D_3$ for 24 h abolished the inhibitory effect of the hormone on [^{3}H]-uridine incorporation (Fig. 9B). Pretreatment with 10^{-7} *M* 24,25-$(OH)_2D_3$ for 24 h caused an increase in [^{3}H]-uridine incorporation. The stimulation was biphasic and significant at 10^{-10}–10^{-7} *M* 17β (Fig. 9C). Pretreatment with 10^{-8} or 10^{-7} *M* 24,25-$(OH)_2D_3$ for 48 h did not influence the response of these cells with respect to [^{3}H]-uridine incorporation (data not shown).

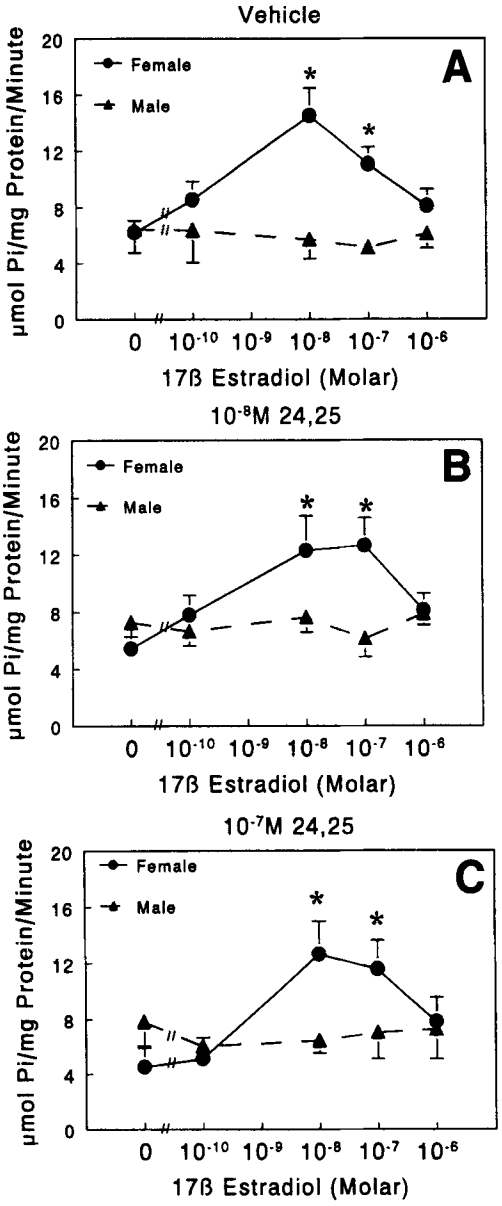

Fig. 7. Effect of pretreatment with 24,25-$(OH)_2D_3$ for 48 h on ALPase of RC in response to 17β. Confluent, fourth-passage cultures of RC from male and female rats were pretreated with control vehicle (**A**), 10^{-8} *M* 24,25-$(OH)_2D_3$ (**B**), or 10^{-7} *M* 24,25-$(OH)_2D_3$ (**C**) for 48 h. At the end of pretreatment, the media were replaced with fresh media containing 10^{-10}–10^{-6} *M* 17β, the incubation continued for another 24 h, and ALPase in the cell layer measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments. $^*P < 0.05$, treatment vs control.

[^{35}S]-Sulfate Incorporation

17β (10^{-10}–10^{-7} *M*) stimulated [^{35}S]-sulfate incorporation by female GC chondrocytes (Fig. 10A). Pretreatment with 10^{-9} *M* 1,25-$(OH)_2D_3$ for 24 h had no effect on this response (Fig. 10B). Pretreatment with 10^{-8} *M* 1,25-$(OH)_2D_3$ raised the level of sulfation in the control cultures (i.e., no 17β) to levels comparable to those seen in cultures treated with 10^{-10}–10^{-7} *M* 17β alone. No further effect of 17β was noted in the cultures

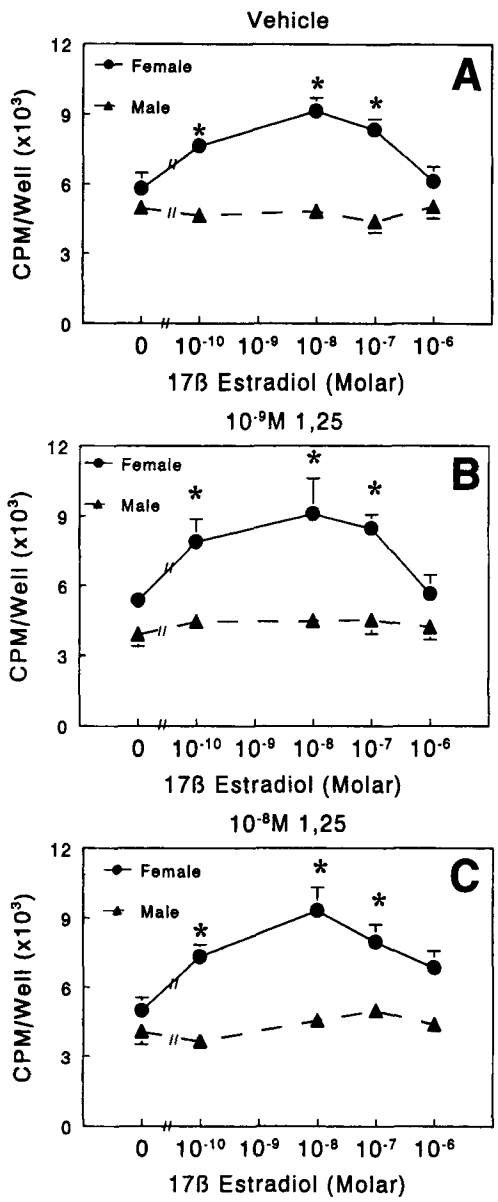

Fig. 8. Effect of pretreatment with 1,25-$(OH)_2D_3$ for 24 h on [^{3}H]-uridine incorporation by GC in response to 17β. Confluent, fourth-passage cultures of GC from male and female rats were pretreated with control vehicle **(A)**, 10^{-9} *M* 1,25-$(OH)_2D_3$ **(B)**, or 10^{-8} *M* 1,25-$(OH)_2D_3$ **(C)** for 24 h. At the end of pretreatment, the media were replaced with fresh media containing 10^{-10}–10^{-6} *M* 17β, the incubation continued for another 24 h, and [^{3}H]-uridine incorporation by the cells was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments. *$P < 0.05$, treatment vs control.

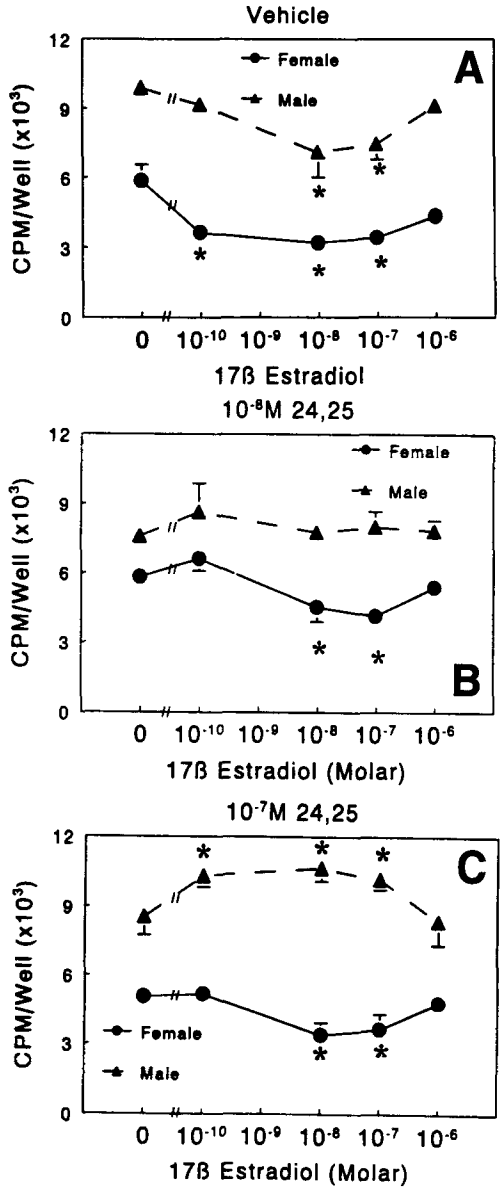

Fig. 9. Effect of pretreatment with 24,25-$(OH)_2D_3$ for 24 h on [^{3}H]-uridine incorporation by RC in response to 17β. Confluent, fourth-passage cultures of RC from male and female rats were pretreated with control vehicle **(A)**, 10^{-8} *M* 24,25-$(OH)_2D_3$ **(B)**, or 10^{-7} *M* 24,25-$(OH)_2D_3$ **(C)** for 24 h. At the end of pretreatment, the media were replaced with fresh media containing 10^{-10}–10^{-6} *M* 17β, the incubation continued for another 24 h, and [^{3}H]-uridine incorporation by the cells was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments. *$P < 0.05$, treatment vs control.

treated with 10^{-8} *M* 1,25-$(OH)_2D_3$ (Fig. 10C). [^{35}S]-Sulfate incorporation by male GC cells was unaffected by 17β (Fig. 10A) whether or not the cultures were pretreated with 1,25-$(OH)_2D_3$ (Fig. 10B,C).

There were no significant changes in [^{35}S]-sulfate incorporation by female or male RC cells after treatment with 17β. Pretreatment with 10^{-8} or 10^{-7} *M* 24,25-$(OH)_2D_3$ for both 24 and 48 h also had no effect on cellular response to 17β (data not shown).

Discussion

The results of this study show that pretreatment with 1,25-$(OH)_2D_3$ augmented the inhibitory effect of 17β on [^{3}H]-thymidine incorporation, abolished the stimulatory effect of 17β on alkaline phosphatase activity, and abolished the stimulatory effect of 17β on [^{35}S]-sulfate incorporation in female GC cultures. Similar results have been described in cultured UMR106 cells incubated with a combination of 17β and 1,25-$(OH)_2D_3$ *(7)*. Other researchers

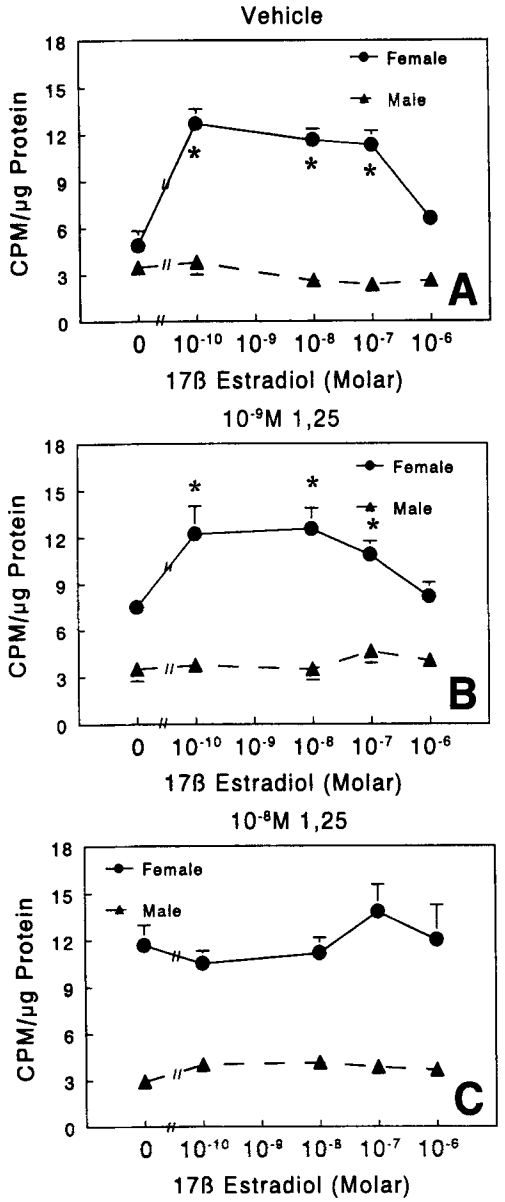

Fig. 10. Effect of pretreatment with 1,25-$(OH)_2D_3$ for 24 h on [^{35}S]-sulfate incorporation by GC in response to 17β. Confluent, fourth-passage cultures of GC from male and female rats were pretreated with control vehicle (**A**), 10^{-9} *M* 1,25-$(OH)_2D_3$ (**B**), or 10^{-8} *M* 1,25-$(OH)_2D_3$ (**C**) for 24 h. At the end of pretreatment, the media were replaced with fresh media containing 10^{-10}–10^{-6} *M* 17β, the incubation continued for another 24 h, and [^{35}S]-sulfate incorporation by the cells was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments. *$P < 0.05$, treatment vs control.

have shown that pretreatment with 1,25-$(OH)_2D_3$ augmented the 17β effect on [^{3}H]-thymidine incorporation in rat epiphyseal cartilage cell cultures *(27,35)* and ROS 17/2.8 cell cultures *(36)*. This suggests that the interrelationship between 1,25-$(OH)_2D_3$ and 17β is consistent for calcifying cells of mesenchymal origin.

Our results also show that the effects of pretreatment with 1,25-$(OH)_2D_3$ on cellular response to 17β are sex-specific. GC derived from male rats did not respond to 17β. The lack of response was not altered by pretreatment with 1,25-$(OH)_2D_3$, even though our previous studies have shown that both male and female cells are regulated by this vitamin D metabolite (Schwartz et al., unpublished observations).

The interactions between 17β and 1,25-$(OH)_2D_3$ observed in this study are, therefore, both interesting and puzzling. The effects of 1,25-$(OH)_2D_3$ on the response of GC to 17β depend on the concentration of 1,25-$(OH)_2D_3$ used and the length of exposure. For example, a 24-h exposure to 1,25-$(OH)_2D_3$ had no effect on 17β-dependent inhibition of [^{3}H]-thymidine incorporation by female growth zone cells, but after 48 h, 1,25-$(OH)_2D_3$ increased the inhibitory effect of 17β. In our previous studies, we showed that treatment with 1,25-$(OH)_2D_3$ for 24 h promotes GC to express a more differentiated phenotype, particularly with respect to ALPase *(47)* and [^{35}S]-sulfate incorporation. The results of the current study indicate that longer exposures may further the differentiation process, since female growth zone cells exposed to 1,25-$(OH)_2D_3$ for 48 h lose this ability to respond to 17β.

This hypothesis is supported by the experiments in which male RC were pretreated with 24,25-$(OH)_2D_3$ prior to treatment with 17β. We have previously shown that exposure of confluent resting zone cells to 24,25-$(OH)_2D_3$ for 36–48 h causes a shift in phenotypic expression of these cells to a 1,25-$(OH)_2D_3$-responsive cell typical of those isolated from growth zone cartilage *(54)*. After 24 h, pretreatment of the male resting zone cells with 24,25-$(OH)_2D_3$ had no effect on the 17β-dependent inhibition of [^{3}H]-thymidine incorporation, but after 48 h, 24,25-$(OH)_2D_3$ caused the cells to exhibit increased [^{3}H]-thymidine incorporation in response to 17β. The levels of [^{3}H]-thymidine incorporation achieved are comparable to those seen in male cells at baseline. We did not challenge these cells with 1,25-$(OH)_2D_3$ to see if they had become responsive, however. Except for [^{3}H]-uridine incorporation, the effects of 17β in the present study are not cell maturation-specific, as has been noted previously *(11–19)*. Previous studies indicated that the effects of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ alone, at least for the parameters tested, also were not sex-specific. However, the present study indicates that after RC and GC are exposed to their target cell-specific vitamin D metabolite, cell maturation specificity to the 17β response is conferred. This is in agreement with other researchers who have found that vitamin D deficiency voids the 17β effect in female rats *(27)*.

The mechanisms by which this occurs are not clear. One possibility is that genes for estrogen receptors or their post-translational modification are regulated, such that the number of receptors may be increased or decreased, and the response to the hormone correspondingly altered. In fact, estrogen receptor levels in male chondrocytes are significantly less than in female cells *(22)*. In the present study, we did not examine this directly. However, 1,25-$(OH)_2D_3$ has been shown to increase estrogen receptors in bone explants *(55)* and in bone marrow-derived stromal cell lines *(37)*.

Moreover, the presence of 1,25-$(OH)_2D_3$ is necessary for the full expression of estrogen receptors in human osteoblast-like cell lines *(56)*.

It needs to be said that although in osteoblasts 1,25-$(OH)_2D_3$ induces the estrogen receptor, in the costochondral GC, the data suggest that it may do the opposite by reducing receptor number. This is in contrast to the recent work by Somjen et al. *(57)*. These investigators showed that pretreatment of ROS 17/2.8 cells and neonatal epiphyseal chondrocytes with 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ caused an increase in creatinine kinase activity and [^{3}H]-thymidine incorporation owing to 17β. These data support the idea that vitamin D metabolites can modify chondrocyte response to 17β; they also show that the nature of the response may vary. For example, in neonatal chondrocytes, pretreatment caused an enhancement of the stimulatory effect of 17β on [^{3}H]-thymidine incorporation, whereas we noted an inhibition. The variation between the two studies may be owing to different model systems used, differences of cell type (neonatal vs mature), and the length of pretreatment (5 vs 1–2 d).

The 1,25-$(OH)_2D_3$ effect may also be mediated through mechanisms that do not involve the traditional estrogen receptor. Vitamin D metabolites have been shown to cause changes in membrane fluidity, fatty acid metabolism, prostaglandin production, calcium ion flux, and protein kinase activity *(58)*. Pretreatment with PGE_2 has been shown to decrease responsiveness of ROS 17/2.8 cells, epiphyseal cartilage, and embryonic calvaria to 17β *(36)*. We have shown that 1,25-$(OH)_2D_3$ stimulates PGE_2 production by GC, whereas 24,25-$(OH)_2D_3$ inhibits production by RC *(52)*. This may explain why pretreatment with 1,25-$(OH)_2D_3$ abolished the response to 17β, whereas 24,25-$(OH)_2D_3$ did not alter 17β-dependent alkaline phosphatase activity.

Both 1,25-$(OH)_2D_3$ *(48)* and 17β in females *(15)* inhibit [^{3}H]-thymidine incorporation by GC. Thus, augmentation of the inhibitory effect of 17β may result from the additive effect of these two factors.

The results of this study indicate that vitamin D metabolites modulate the response of rat costochondral chondrocytes to 17β in vitro. Although the effect of vitamin D metabolites alone on these cells is maturation-dependent *(47–52)*, and not sex-specific, the state of cell differentiation, and especially the sex of the animal from which the cells were derived, will affect the ability of the vitamin D metabolites to modify cellular response to the hormone. The mechanisms through which vitamin D metabolites mediate their effects on cellular response are not clear, and two important questions are raised:

1. Do vitamin D metabolites regulate estrogen receptors in these chondrocytes?
2. Do they mediate their effect on cellular response through genomic or nongenomic mechanisms or both?

Resolution of these questions is presently under way.

Materials and Methods

Chondrocyte Cultures

Chondrocytes were isolated from the resting zone (reserve zone) or growth zone (prehypertrophic and upper hypertrophic zones) of the costochondral cartilages of 125-g male or female Sprague-Dawley rats as previously described *(47,49)*. The cells were plated in 25-mm culture dishes at an initial density of 10,000 cells/cm^2 for RC or 25,000 cells/cm^2 for GC. Cells were incubated in DMEM containing 10% fetal bovine serum (FBS), 50 μg/mL vitamin C, and 1% penicillin-streptomycin in an atmosphere of 5% CO_2 at 37°C and 100% humidity. The culture media were replaced after the first 24 h and then at 72-h intervals. Fourth-passage cells were used for the experiments because our previous studies have demonstrated that these cells retain their differential phenotype and cell maturation-dependent responsiveness to vitamin D metabolites and growth factors at this passage *(47,49,53,54,59–65)*.

Experimental Design

Fourth-passage resting zone or growth zone cells were grown to confluence. At confluence, the media were replaced, and the resting zone and growth zone cells pretreated for 24 or 48 h with media containing 10^{-8}–10^{-7} *M* 24,25-$(OH)_2D_3$ or 10^{-9}–10^{-8} *M* 1,25-$(OH)_2D_3$, respectively, or vehicle alone (control). At the end of the pretreatment, the media were again replaced and the cultures incubated with 10^{-9}–10^{-8} *M* 17β or vehicle alone (control) for an additional 24 h. Both 17β and the vitamin D metabolites were solubilized in ethanol. The stock solutions were diluted 1:5000 (v/v) with DMEM before addition to the culture media to minimize any toxic effects. Vehicle controls contained ethanol at the highest concentration used for the experimental cultures.

[^{3}H]-Thymidine Incorporation

Fourth-passage chondrocytes were grown to confluence in 6-mm diameter microwells (96-well plate). The chondrocytes were then incubated 24 or 48 h in DMEM containing 10% FBS and either vehicle or the vitamin D metabolite. At the end of that time, the media were replaced with media containing the appropriate concentration of ethanol. [^{3}H]-Thymidine (50 μL) was added 4 h before harvest, so that the final concentration in the medium was 2 μCi/mL. At harvest, the cell layers were washed twice with cold PBS and twice with 5% trichloroacetic acid (TCA), and then treated with saturated TCA for 30 min. TCA-precipitable material was dissolved in 0.2 mL 1% sodium dodecyl sulfate, and the radioactivity was measured by scintillation spectroscopy.

Alkaline Phosphatase Specific Activity

Resting zone and growth zone cells were cultured in 24-well plate culture dishes (Corning Glass Works, Corning, NY), and the enzyme assays performed using lysates of the cell layers *(49,66)*. At harvest, the media were decanted,

and the cell layers washed twice with PBS before removal with a cell scraper. After centrifugation at 500*g*, the cell layer pellet was washed twice with PBS and resuspended by vortexing in 500 µL deionized water containing 25 µL 1% Triton X-100. Alkaline phosphatase (orthophosphoric monoester phosphohydrolase alkaline; EC 3.1.3.1) specific activity was measured as a function of *para*-nitrophenol release from *para*-nitrophenylphosphate at pH 10.2, as previously described *(67)*.

[^{3}H]-Uridine Incorporation

RNA synthesis by resting zone or growth zone cells was estimated by measuring [^{3}H]-uridine incorporation into TCA-insoluble precipitates *(48)*. Fourth-passage chondrocytes were grown to confluence in 6-mm diameter microwells as described above, and [^{3}H]-uridine (50 µL) was added 2 h before harvest, so that the final concentration in the medium was 14 µCi/mL. At harvest, the cell layers were washed twice with cold PBS and twice with 5% TCA, and then treated with saturated TCA for 30 min. TCA-precipitable material was dissolved in 0.2 mL 1% sodium dodecyl sulfate, and the radioactivity measured by scintillation spectroscopy.

[^{35}S]-Sulfate Incorporation

Proteoglycan synthesis was assessed by measuring [^{35}S]-sulfate incorporation according to the method of O'Keefe et al. *(68)*. In prior studies, we found that the amount of radiolabeled proteoglycan released by growth zone and resting zone cells into the medium was <15% of the total radiolabeled proteoglycan (medium and cell layer) synthesized *(22)*. Because of this, we only examined the effects of hormone treatment on [^{35}S]-sulfate incorporation in the cell layer (into the matrix). This assay does not measure any degradation that may occur during the labeling period.

For assay, fourth-passage resting zone or growth zone cells were grown to confluence in 24-well culture plates (Corning), and 50 µL [^{35}S]-sulfate containing 18 µCi/mL and 0.814 m*M* carrier sulfate were added 4 h before harvest. At harvest, the conditioned media were removed and the cell layers (cells and matrix) collected in two 0.25-mL portions of 0.25 *M* NaOH. The protein content was determined by the method of Lowry et al. *(69)*. The total volume was adjusted to 0.75 mL by the addition of 0.15 *M* NaCl and the sample dialyzed in a 12,000–14,000 mol wt cutoff membrane against buffer containing 0.15 *M* NaCl, 20 m*M* Na_2SO_4, and 20 m*M* Na_2HPO_4, pH 7.4, at 4°C. The dialysis solution was changed until the radioactivity in the dialysate reached background. The amount of [^{35}S]-sulfate incorporated was determined by liquid scintillation spectrometry and calculated as disintegrations/min/mg protein in the cell layer.

Statistical Analysis

The data presented here are from one of three or more replicate experiments. For any given experiment, each data point represents the mean ± SEM for six individual cultures (cell layers). Treatment/control ratios were derived from five or more independent experiments, with controls having a ratio of 1.0.

The data were analyzed by analysis of variance and statistical significance determined by comparing each data point to the control (containing the ethanol vehicle) using Bonferroni's *t*-test. Treatment/control ratios were compared using the Wilcoxon matched pair rank sum test. $P<0.05$ was considered significant.

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