17β-Estradiol-BSA Conjugates and 17β-Estradiol Regulate Growth Plate Chondrocytes by Common Membrane Associated Mechanisms Involving PKC Dependent and Independent Signal Transduction

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Abstract Nuclear receptors for 17β-estradiol (E_2) are present in growth plate chondrocytes from both male and female rats and regulation of chondrocytes through these receptors has been studied for many years; however, recent studies indicate that an alternative pathway involving a membrane receptor may also be involved in the cell response. E_2 was found to directly affect the fluidity of chondrocyte membranes derived from female, but not male, rats. In addition, E_2 activates protein kinase C (PKC) in a nongenomic manner in female cells, and chelerythrine, a specific inhibitor of PKC, inhibits E_2-dependent alkaline phosphatase activity and proteoglycan sulfation in these cells, indicating PKC is involved in the signal transduction mechanism. The aims of the present study were: (1) to examine the effect of a cell membrane-impermeable 17β-estradiol-bovine serum albumin conjugate (E_2-BSA) on chondrocyte proliferation, differentiation, and matrix synthesis; (2) to determine the pathway that mediates the membrane effect of E_2-BSA on PKC; and (3) to compare the action of E_2-BSA to that of E_2. Confluent, fourth passage resting zone (RC) and growth zone (GC) chondrocytes from female rat costochondral cartilage were treated with 10^{-9} to 10^{-7} M E_2 or E_2-BSA and changes in alkaline phosphatase specific activity, proteoglycan sulfation, and [^{3}H]-thymidine incorporation measured. To examine the pathway of PKC activation, chondrocyte cultures were treated with E_2-BSA in the presence or absence of GDPβS (inhibitor of G-proteins), GTPγS (activator of G-proteins), U73122 or D609 (inhibitors of phospholipase C [PLC]), wortmannin (inhibitor of phospholipase D [PLD]) or LY294002 (inhibitor of phosphatidylinositol 3-kinase). E_2-BSA mimicked the effects of E_2 on alkaline phosphatase specific activity and proteoglycan sulfation, causing dose-dependent increases in both RC and GC cell cultures. Both forms of estradiol inhibited [^{3}H]-thymidine incorporation, and the effect was dose-dependent. E_2-BSA caused time-dependent increases in PKC in RC and GC cells; effects were observed within three minutes in RC cells and within one minute in GC cells. Response to E_2 was more robust in RC cells, whereas in GC cells, E_2 and E_2-BSA caused a comparable increase in PKC. GDPβS inhibited the activation of PKC in E_2-BSA-stimulated RC and GC cells. GTPγS increased PKC in E_2-BSA-stimulated GC cells, but had no effect in E_2-BSA-stimulated RC cells. The phosphatidylinositol-specific PLC inhibitor U73122 blocked E_2-BSA-stimulated PKC activity in both RC and GC cells, whereas the phosphatidylcholine-specific PLC inhibitor D609 had no effect. Neither the PLD inhibitor wortmannin nor the phosphatidylinositol 3-kinase inhibitor LY294022 had any effect on E_2-BSA-stimulated PKC activity in either RC or GC cells. The classical estrogen receptor antagonist ICI 182780 was unable to block the stimulatory effect of E_2-BSA on PKC. Moreover, the classical receptor agonist diethylstilbestrol (DES) had no effect on

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PKC, nor did it alter the stimulatory effect of E_2-BSA. The specificity of the membrane response to E_2 was also demonstrated by showing that the membrane receptor for $1\alpha,25$-$(OH)_2D_3$ was not involved. These data indicate that the rapid nongenomic effect of E_2-BSA on PKC activity in RC and GC cells is dependent on G-protein-coupled PLC and support the hypothesis that many of the effects of E_2 involve membrane-associated mechanisms independent of classical estrogen receptors. J. Cell. Biochem. 81:413–429, 2001. © 2001 Wiley-Liss, Inc.

Key words: chondrocyte cultures; 17β-estradiol-BSA; protein kinase C; signal transduction; phospholipase C; G-proteins

17β-Estradiol (E_2) regulates endochondral bone formation through direct action on chondrocytes and indirectly through secretion of other hormones and growth factors. Growth plate chondrocytes possess nuclear receptors for E_2 [Nasatzky et al., 1994a], but some of the effects of E_2 on growth plate chondrocytes are mediated by rapid, membrane-associated mechanisms [Schwartz et al., 1996; Sylvia et al., 1998a]. Using rat costochondral chondrocytes as a model for studying agents which modulate cartilage cell differentiation and maturation [Boyan et al., 1988a], we have shown that E_2 causes a direct increase in arachidonic acid turnover, phospholipase A_2 activity, and membrane fluidity in cultures of chondrocytes derived from female, but not male, rats [Schwartz et al., 1996]. Additionally, E_2 stimulates protein kinase C (PKC) activity in a dose-dependent manner via mechanisms that are independent of new gene expression [Sylvia et al., 1998a].

These rapid events may be due to a unique membrane receptor for the hormone that is structurally distinct from the nuclear receptor [Pappas et al., 1994; Ramirez et al., 1996]. Our studies support this hypothesis. We have shown that E_2 affects fluidity of matrix vesicles isolated from female, but not male, chondrocyte cultures [Schwartz et al., 1996]. Since these extracellular organelles contain no DNA or the ability to synthesize new proteins, the effect of E_2 is purely on the membrane. 17α-Estradiol does not affect membrane fluidity, nor does it affect PKC activity, demonstrating that the response to E_2 is stereo-specific, as is typical of receptor-mediated mechanisms.

There is a growing body of evidence that membrane-mediated effects of steroid hormones proceed via pathways traditionally ascribed to peptide hormones, including PKC [Pailler-Rodde et al., 1991; Magda and Lloyd, 1993; Morelli et al., 1996; Wehling, 1997] and MAP kinase [Endoh et al., 1997]. PKC appears to be involved in the biological response to E_2,

since PKC inhibitors block the action of this steroid on DNA synthesis [Rajkumar, 1993; Fujimoto et al., 1996]. Inhibition of PKC with chelerythrine also blocks the effects of E_2 on chondrocyte differentiation and proteoglycan sulfation, but it does not affect E_2-dependent changes in proliferation [Sylvia et al., 2000]. Moreover, this effect of E_2 on PKC in female rat growth plate chondrocytes is mediated via a G-protein-dependent phosphatidylinositol-specific phospholipase C (PI-PLC).

It is possible that the rapid effects elicited by E_2 are not due to a membrane-associated receptor, however, since E_2 can readily traverse the plasma membrane and bind its traditional receptor ERα and/or the more recently identified ERβ [Kuiper et al., 1996]. Moreover, the ERα receptor has been identified at the plasma membrane of Chinese hamster ovary cells [Razandi et al., 1999], suggesting that it may serve as a docking mechanism for the hormone. The effect of E_2 on PKC does not appear to be due to classic E_2 receptors, at least in resting zone chondrocytes, since neither diethylstilbesterol nor ICI-182780 affected E_2-stimulated activity [Sylvia et al., 2000].

Studies using E_2-bovine serum albumin (BSA) conjugates suggest that it may not be necessary for E_2 to enter the cell to initiate membrane-associated mechanisms [Benten et al., 1998; Stefano et al., 1999]. Zheng et al. [1996] confirmed that E_2-BSA remains extracellular by showing that rat brain cells would bind to columns to which E_2-BSA was covalently attached. In this study, we used E_2-BSA to test the hypothesis that E_2 exerts its effects on proliferation, differentiation, and matrix synthesis of growth plate chondrocytes via membrane-associated mechanisms that are mediated by the PKC signaling pathway. We examined the effects of E_2-BSA and E_2 on [^{3}H]-thymidine incorporation, alkaline phosphatase specific activity, and proteoglycan sulfation in resting zone and growth zone chondrocyte cultures. To determine the pathway mediating the effect of

E_2-BSA on PKC, we inhibited the actions of G-proteins, PLC, phospholipase D (PLD), and cyclooxygenase (Cox). In addition, we examined the potential contribution of the nuclear estrogen receptors by determining the effects of estrogen receptor agonists and antagonists on PKC activity in E_2-BSA-treated cultures and used a specific antibody to the $1,25$-$(OH)_2D_3$ membrane receptor to demonstrate that the membrane-dependent effect on PKC activity was specific for E_2.

MATERIALS AND METHODS

Reagents

17β-estradiol (E_2), 17β-estradiol-BSA (E_2-BSA), diethylstilbesterol (DES), GDPβS, GTP-γS, and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO). The estrogen receptor antagonist ICI 182780 [Wakeling, 1995] was obtained from Tocris Cookson, Inc. (Ballwin, MO). The following chemicals were purchased from Calbiochem (San Diego, CA): U73122 (PI-PLC inhibitor) [Bleasdale et al., 1989], D609 (phosphatidylcholine-specific PLC [PC-PLC] inhibitor) [Muller-Decker, 1989], LY294002 (phosphatidylinositol 3-kinase (PI3-kinase] inhibitor) [Vlahos et al., 1994], and nordihydroguaiaretic acid (NDGA; 5-lipoxygenase inhibitor) [Hope et al., 1983]. PKC assay reagents and Dulbecco's modified Eagle medium (DMEM) were obtained from GIBCO-BRL (Gaithersburg, MD). The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay reagent [Smith et al., 1985] obtained from Pierce Chemical Co. (Rockford, IL). $[^{32}P]$-ATP, $[^3H]$-thymidine and $[^{35}S]$-sulfate were obtained from NEN-DuPont (Boston, MA).

Chondrocyte Cultures

The culture system used in this study has been described in detail previously [Boyan et al., 1988b]. Chondrocytes were isolated from the resting zone (RC; reserve zone) and growth zone (GC; prehypertrophic/upper hypertrophic cell zones) of the costochondral junction of 125g female Sprague-Dawley rats by enzymatic digestion and cultured in DMEM containing 10% fetal bovine serum (FBS) and 50 μg/ml vitamin C in an atmosphere of 5% CO_2 and 100% humidity at 37°C for 24 h. We previously showed that the membrane-dependent effects of E_2 were observed in the presence of FBS and that, in the absence of FBS, the chondrocyte cultures fail to grow [Schwartz et al., 1996; Sylvia et al., 1998a]. Fourth passage cells were used for all experiments. Previous studies have shown that these cells retain their chondrogenic phenotype, including the ability to form cartilage nodules when implanted in nude mouse thigh muscle [Boyan et al., 1992a]. Furthermore, they retain their differential responsiveness to vitamin D metabolites at this passage [Schwartz et al., 1989], as well as differential responsiveness to a number of other factors [Boyan et al., 1988b, 1992b, 1997].

Since normal serum estradiol ranges from 10^{-9} to 10^{-8} M, we chose to test E_2-BSA at concentrations ranging from 10^{-9} to 10^{-7} M. Both E_2 and E_2-BSA were dissolved in absolute ethanol and diluted in culture medium by at least 1,000-fold to the required concentration. Control cultures contained ethanol at the highest concentration used in the experimental cultures.

Chondrocyte Response to E_2-BSA

$[^3H]$-Thymidine incorporation. E_2 inhibits $[^3H]$-thymidine incorporation by RC and GC chondrocytes [Nasatzky et al., 1993]; therefore, we determined whether E_2-BSA elicits a similar response. DNA synthesis was assessed by measuring $[^3H]$-thymidine incorporation into trichloroacetic acid (TCA) insoluble cell precipitates as described previously [Schwartz et al., 1989]. Quiescence was induced by incubating confluent cultures of female RC or GC cells for 48 h in DMEM containing 1% FBS. The medium was then replaced with DMEM containing 1% FBS alone (control), or with control medium containing 10^{-9}–10^{-7} M E_2 or E_2-BSA for 24 h. Two hours prior to harvest, $[^3H]$-thymidine was added.

Alkaline phosphatase specific activity. E_2 has been shown previously to stimulate alkaline phosphatase specific activity in both RC and GC chondrocyte cultures [Nasatzky et al., 1993]. To examine the effect of E_2-BSA on this parameter, confluent cells were treated with medium containing vehicle alone or 10^{-9}–10^{-7} M E_2 or E_2-BSA for 24 h. Alkaline phosphatase [orthophosphoric monoester phosphohydrolase, alkaline (EC 3.1.3.1)] specific activity was measured in cell layer lysates as a function of release of para-nitrophenol from para-nitrophenylphosphate at pH 10.2 [Bretaudiere and Spillman, 1984; Hale et al., 1986]. Protein

content of the cell layer was determined according to the method of Smith et al. [1985].

Proteoglycan sulfation. E_2 stimulates proteoglycan sulfation in RC and GC chondrocyte cultures [Nasatzky et al., 1994b]. To determine whether E_2-BSA elicits a similar response, E_2 and E_2-BSA were added to both female GC and RC cells and proteoglycan synthesis was assessed by measuring [^{35}S]-sulfate incorporation by confluent cultures as described previously [Nasatzky et al., 1994b; O'Keefe et al., 1988]. E_2-BSA (10^{-9}–10^{-7} M) was added to confluent cultures for an additional 24 h. Cells were labeled with [^{35}S]-sulfate four hours prior to harvest. At harvest, the conditioned media were removed, the cell layers (cells and matrix) collected, and the amount of [^{35}S]-sulfate incorporated determined as a function of protein in the cell layer.

Protein Kinase C

To examine the effect of E_2-BSA on PKC activity and determine the signaling pathways involved, the following experimental protocols were used. For each experiment, confluent cultures in 24-well plates were treated for various time periods with 0.5 ml of vehicle control (0.02% ethanol in DMEM + 10% FBS) or experimental DMEM + 10% FBS plus various concentrations of E_2-BSA in the absence or presence of various concentrations of inhibitors as described below. Since E_2 activates PKC in RC from female rats as quickly as 3 min, reaches maximum activity at 90 min, and remains significantly higher than control even after 4 h of treatment [Sylvia et al., 1998b], experimental time points of 1, 3, 9, 30, 90 and 270 min were chosen. In one set of experiments, female RC and GC cultures were treated for 90 min with 10^{-8}–10^{-7} M E_2, E_2-BSA, or the two in combination. To ensure that any effects of E_2-BSA on PKC were not due to the 0.3% free E_2 (3 μg E_2/mg E_2-BSA) reported to be present in E_2-BSA preparations [Stevis et al., 1999], media containing 10^{-9} or 10^{-8} M E_2-BSA were filtered through a 3,000-μm filter (Mircrocon YM-3 centrifugal filter units, Millipore, Bedford, MA). Confluent cultures were treated with retentate containing E_2-BSA or with filtrate containing E_2.

After the appropriate incubation period, cell layers were washed with phosphate buffered saline (PBS) and lysed in solubilization buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride and 1% NP-40) for 30 min on ice. The cell layer lysates were assayed for protein content [Smith et al., 1985] and PKC activity [Sylvia et al., 1993]. Cell layer lysates were mixed for 20 min with a lipid preparation containing phorbol-12-myristate-13-acetate, phosphatidylserine, and Triton X-100 mixed micelles, which provides the necessary cofactors and conditions for optimal activity [Bell et al., 1986]. To this mixture, a high affinity myelin basic protein peptide substrate and [^{32}P]-ATP (25 μCi/ml) were added to a final volume of 50 μl. Following a 10-minute incubation at 30°C, samples were spotted onto phosphocellulose disks, washed, and counted in a scintillation counter.

G-proteins. G-proteins mediate the effect of E_2 on PKC in RC cells; however, neither Gi nor Gs is involved [Sylvia et al., 2000]. To further assess the role of G-proteins, the nonhydrolyzable inhibitor GDPβS and activator GTPγS were used. These factors have the ability to affect intracellular G-proteins of intact cells in culture when added in the media rather than via microinjection or cell permeabilization [Rodriguez et al., 1992; Boonen et al., 1993; Bominaar and Van Haastert, 1994]. Female RC or GC cell cultures were treated for 90 min with control media, 10^{-8} M E_2 or 10^{-8} M E_2-BSA in the presence or absence of 1 or 10 μM GDPβS or GTPγS, and PKC activity determined.

Phospholipase C. PLC mediates the effects of E_2 on PKC in RC cell cultures [Sylvia et al., 2000]. Its involvement in the membrane-mediated effects of E_2-BSA on PKC was assessed using U73122, an inhibitor of PI-PLC [Bleasdale et al., 1989], and D609, an inhibitor of PC-PLC [Muller-Decker, 1989]. Cultures were incubated for 90 min in media containing 10^{-8} M E_2-BSA plus 1 or 10 μM U73122 or with 10 or 100 μM D609, and PKC activity was determined. Controls contained vehicles used for E_2-BSA and the inhibitors.

Phospholipase D. PLD mediates the membrane effects of the seco-steroid 24R,25-$(OH)_2D_3$ on PKC activity of RC cells, but not GC cells [DeVeau et al., 2000]. Its involvement in the membrane-mediated effects of E_2 and E_2-BSA on PKC activity was assessed using wortmannin, an inhibitor of PLD [Carrasco-Marin et al., 1994; Mollinedo et al., 1994]. RC and GC cells were treated with 0.1, 1, or 10 μM wortmannin. Because wortmannin also inhibits PI 3-kinase and PI 3-kinase may also play a role in regulating PKC, the effects of E_2 and E_2-BSA

on PKC activity were also determined in the presence of the PI 3-kinase specific inhibitor LY294022 (10 μM) [Vlahos et al., 1994].

Cyclooxygenase and lipooxygenase. E_2 was shown previously not to activate PKC through a mechanism involving phospholipase A_2 or through metabolites of arachidonic acid [Sylvia et al., 2000], the product of phospholipase A_2 action, suggesting that the membrane-dependent actions of E_2 do not involve these pathways. To test this hypothesis, RC and GC cells were treated for 90 min with control media or 10^{-8} M E_2 or E_2-BSA in the presence or absence of the general Cox inhibitor indomethacin (10^{-8}–10^{-6} M) [Vane et al., 1998]. To examine the role of lipooxygenase (LPX), the inhibitor NDGA (2, 20 or 40 μM) [Hope et al., 1983] was used.

Role of Classical Estrogen Receptors

To assess the role of classical estrogen receptors in the effect of E_2-BSA on PKC, female RC or GC cultures were treated with E_2-BSA in the presence or absence of estrogen receptor agonist DES or antagonist ICI 182780. RC cultures were treated for 90 minutes with control media (DMEM + 10% FBS) or media containing 10^{-9}–10^{-7} M DES or ICI 182780 in the presence or absence of 10^{-9}–10^{-7} M E_2-BSA. In a separate set of experiments, 1 μM and 10 μM ICI 182780 were also tested.

Relationship to Membrane Receptors for $1\alpha,25$-$(OH)_2D_3$

Membrane receptors have been reported for a number of steroid hormones (see Nemere and Farach-Carson [1998] for a review). In the rat chondrocyte model used in the present study, the vitamin D_3 metabolite $1\alpha,25$-$(OH)_2D_3$ has been shown to exert its membrane-dependent effects via PKC. PKC activity in GC cells is increased by treatment with $1\alpha,25$-$(OH)_2D_3$, and maximal response is observed at 9 minutes [Sylvia et al., 1993, 1998b]. In contrast, PKC activity in RC is not affected by $1\alpha,25$-$(OH)_2D_3$. Since both $1\alpha,25$-$(OH)_2D_3$ and E_2 exert their physiologic effects via a membrane-associated, PKC-dependent mechanism, we tested the hypothesis that the membrane receptor for $1\alpha,25$-$(OH)_2D_3$ (mVDR) plays a role in E_2 action. To determine whether E_2-BSA interacts with the mVDR, the ability of the anti-mVDR antibody (Ab99) [Nemere et al., 1998] to block the effect of E_2-BSA on PKC was examined in female RC and

GC cultures. Cultures were treated with 10^{-8} M $1\alpha,25$-$(OH)_2D_3$ for 9 min or with 10^{-8} M E_2 or E_2-BSA for 90 min, respectively, in the presence or absence of a 1/500 dilution of Ab99 or rabbit immunoglobulin G (nonspecific antibody), prior to determination of PKC activity.

Statistical Management of Data

For each experiment, each value represents the mean ± SEM of the cell layers of six individual independent cultures. Significance between groups was determined by Bonferroni's modification of Student's t-test using $P < 0.05$. Each experiment was repeated two or more times to ensure validity of the data. The data presented are from a single representative experiment.

RESULTS

Chondrocyte Response to E_2-BSA

[³H]-Thymidine incorporation. DNA synthesis by chondrocytes was regulated by E_2-BSA in the same manner as seen with E_2 (Fig. 1). [³H]-Thymidine incorporation was inhibited by both E_2 and E_2-BSA in both RC and GC cells from female rats. The effect of E_2 and E_2-BSA in RC cells was to inhibit [³H]-thymidine incorporation in a dose-dependent manner, with significant decreases at 10^{-8} M and the maximal decrease in cultures treated with 10^{-7} M hormone (Fig. 1A). Similarly, in GC cells, both E_2 and E_2-BSA inhibited DNA synthesis and maximal inhibition was seen at 10^{-7} M hormone (Fig. 1B).

Alkaline phosphatase specific activity. Alkaline phosphatase specific activity was increased by E_2 and E_2-BSA in RC and GC cells in a dose-dependent manner (Fig. 2). The effects of both agents were comparable with maximal stimulation in RC and GC chondrocyte cultures treated with 10^{-7} M (44 and 50%, respectively).

Proteoglycan sulfation. Both E_2 and E_2-BSA caused dose-dependent increases in [³⁵S]-sulfate incorporation in RC and GC chondrocyte cultures. Maximal increases were seen at 10^{-7} M in GC chondrocyte cultures (Fig. 3), as well as in RC chondrocyte cultures (data not shown).

Protein Kinase C

PKC activity was increased in a time-dependent manner by E_2-BSA (Fig. 4). In RC cells, no

Effect of 17β-Estradiol-BSA on [³H]-Thymidine Incorporation

Female RC Cells

Effect of 17β-Estradiol-BSA on Alkaline Phosphatase Activity

Female RC Cells

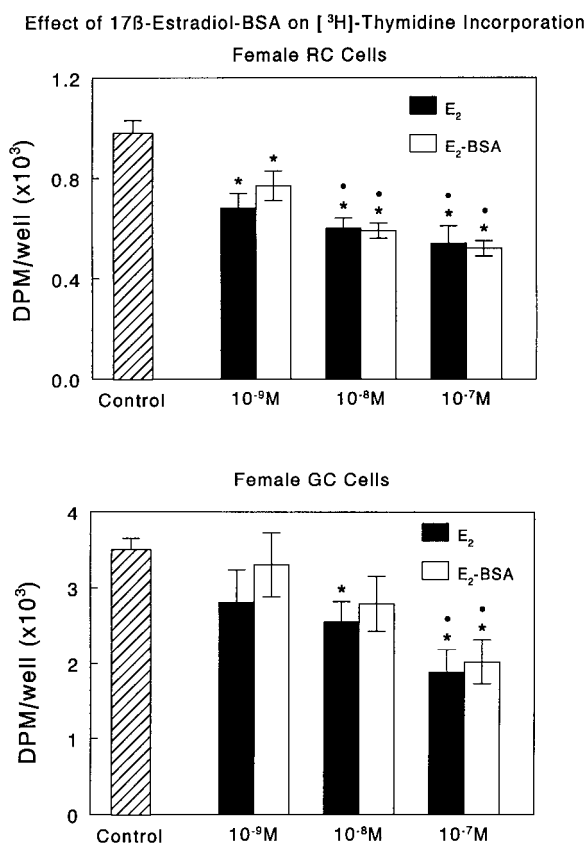**Female GC Cells**

Female GC Cells

Fig. 1. Effect. of 17β-estradiol (E_2) or 17β-estradiol-BSA (E_2-BSA) on [³H]-thymidine incorporation. Confluent, fourth passage resting zone (RC, upper panel) and growth zone (GC, lower panel) chondrocytes from female rats were treated for 24 h with control media or media containing 10^{-9}–10^{-7} M E_2 or E_2-BSA. Two hours prior to harvest, [³H]-thymidine was added to the cultures. At harvest, the cell layers were washed, precipitated with trichloroacetic acid as described in the Methods, and counted in a scintillation counter. Values are the mean-$\pm$ SEM of six cultures from one of three experiments, all with comparable results. *$P < 0.05$, treatment vs control; •$P < 0.05$, vs 10^{-9} M.

Fig. 2. Effect of 17β-estradiol (E_2) or 17β-estradiol-BSA (E_2-BSA) on alkaline phosphatase specific activity. Confluent, fourth passage resting zone (RC, upper panel) and growth zone (GC, lower panel) chondrocytes from female rats were treated for 24 h with control media or media containing 10^{-9}–10^{-7} M E_2 or E_2-BSA. At harvest, alkaline phosphatase specific activity in the cell layer was determined as described in the Methods. Each data point is the mean $\pm$ SEM of six cultures from one of three experiments, all with comparable results. *$P < 0.05$, treatment vs control; •$P < 0.05$, vs 10^{-9} M.

change in PKC activity was evident at 1 min. However, by 3 min, PKC activity was significantly increased in RC cells treated with 10^{-7} M E_2-BSA, and it continued to increase through 90 min (Fig. 4A). By 270 min, PKC activity had returned to the level of control cultures. In GC cells, E_2-BSA increased PKC specific activity within 1 min, and activity continued to increase up through 90 min (Fig. 4B). The rate of increase in PKC activity between 9 and 30 min was greater in GC cells than in RC cells. However, by 270 min, PKC activity had returned to the level of control cultures. Moreover, activity was still increasing at 90 minutes in RC cells, whereas activity had plateaued in GC cells at 90 min.
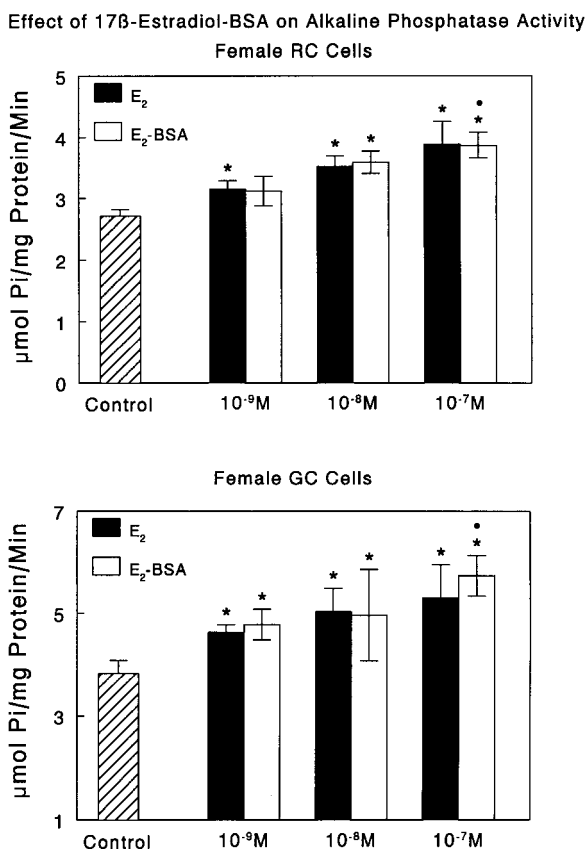
The effects of E_2 and E_2-BSA on PKC specific activity were similar in that both hormones caused a dose-dependent increase in PKC activity in RC cells and in GC cells (Fig. 5). However, in RC cells, the stimulatory effects of E_2 were greater than those of E_2-BSA (Fig. 5A). When RC cells were treated with E_2 and E_2-BSA together, the effect was comparable to that of E_2 alone. In contrast, the effect of E_2 or E_2-BSA on PKC activity in growth zone cells was comparable (Fig. 5B). Moreover, at 10^{-8} M, PKC activity was higher in GC cells treated with E_2 and E_2-BSA in combination than in cultures treated with either agent alone. This small, but significant, increase in activity was not evident in cultures treated with 10^{-7} M E_2 plus E_2-BSA.
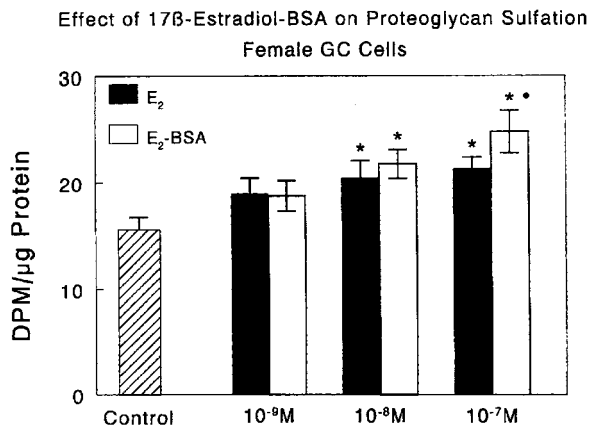
Effect of 17ß-Estradiol-BSA on Proteoglycan Sulfation
Female GC Cells

Fig. 3. Effect of 17β-estradiol (E_2) or 17β-estradiol-BSA (E_2-BSA) on proteoglycan sulfation. Confluent, fourth passage growth zone cells (GC) from female rats were treated for 24 h with control media or media containing 10^{-9}–10^{-7} M E_2 or E_2-BSA. At harvest, proteoglycan sulfation in the cell layer was determined as described in the Methods. Each data point is the mean ± SEM of six cultures from one of three experiments, all with comparable results. *$P < 0.05$, treatment vs control; •$P < 0.05$, vs 10^{-9} M.

The effects of E_2-BSA on PKC were not due to free E_2 in the E_2-BSA preparation (Table I). RC cells treated with the retentate of medium containing 10^{-8} M E_2-BSA exhibited the same increase in PKC specific activity as cultures treated with unfiltered medium. Moreover, the effect of the E_2-BSA retentate was dose-dependent. In contrast, the filtrate had no effect. This was not due to loss of nutrients or co-factors during the filtration process, however. PKC activity in cultures treated with control medium, the retentate of control medium, or the filtrate of control medium was comparable.

Role of G-proteins. G-proteins mediated the effect of both E_2 and E_2-BSA on PKC. The nonhydrolyzable G-protein inhibitor GDPβS had no effect on basal PKC activity, but blocked E_2-stimulated PKC activity in both RC and GC cells (Fig. 6). In RC cells treated with 10^{-8} M E_2, 1 μM GDPβS inhibited PKC activity by >90%, and 10 μM GDPβS inhibited E_2-stimulated activity completely (Fig. 6A). In GC cells treated with 10^{-8} M E_2, 1 μM GDPβS inhibited PKC activity by 95%, and 10 μM inhibited E_2-stimulated activity by 99% (Fig. 6B). E_2-BSA also exerted its effects on PKC in a G-protein-dependent manner (Fig. 7). In RC cells, 10 μM GDPβS caused a 94% decrease in E_2-BSA-stimulated PKC (Fig. 7A). However, in GC cells, GDPβS was a less effective inhibitor of the stimulatory effect of E_2-BSA than was seen in E_2-

Effect of 17ß-Estradiol-BSA on PKC
Female RC Cells

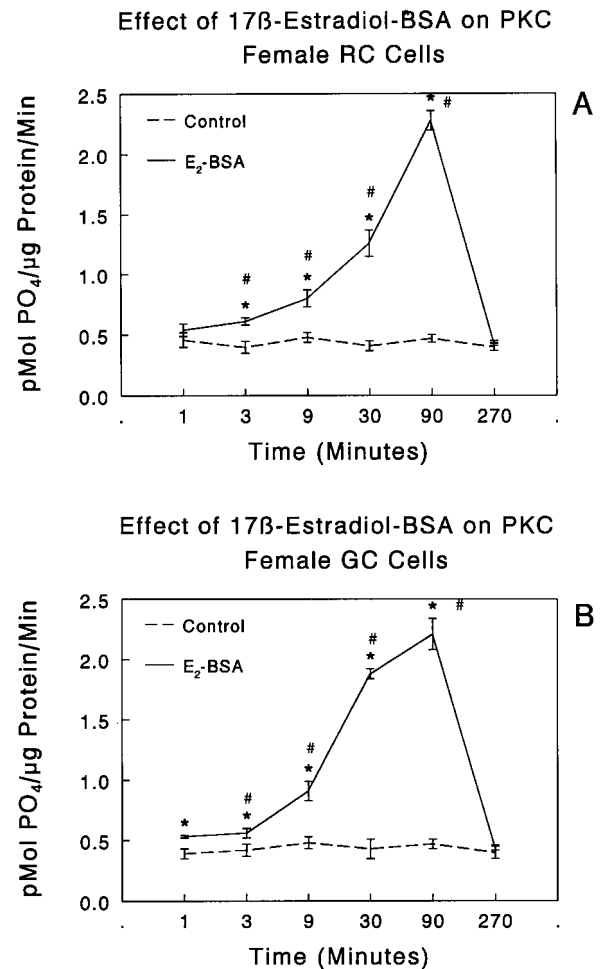Effect of 17ß-Estradiol-BSA on PKC
Female GC Cells

Fig. 4. Time-dependent effect of 17β-estradiol-BSA (E_2-BSA) on PKC specific activity. Confluent, fourth passage resting zone (RC, **Panel A**) or growth zone (GC, **Panel B**) chondrocytes from female rats were treated for 1–270 minutes with control media or media containing 10^{-7} M E_2-BSA. At harvest, PKC specific activity in the cell layer was determined as described in the Methods. Data represent the mean ± SEM of six cultures from one of two experiments yielding comparable results. *$P < 0.05$, E_2-BSA treatment vs control; #$P < 0.05$, treatment vs other time points.

treated cells. In GC cells, 10 μM GDPβS caused a decrease of 58%.

In contrast to the effects of GDPβS, the G-protein activator GTPγS had no effect on PKC activity in control RC cells or in RC cells treated with E_2-BSA (Fig. 8A). However, in GC chondrocytes, there was a small, but significant, increase in basal PKC, but no effect of GTPγS was evident in the E_2-BSA-treated cultures (Fig. 8B).

Role of phospholipase C. PLC was essential for the stimulatory action of E_2-BSA on PKC in RC and GC cells (Fig. 9). In RC cells,

Effect of 17β-Estradiol + E₂-BSA on PKC
Female RC Cells (90 Min)

A

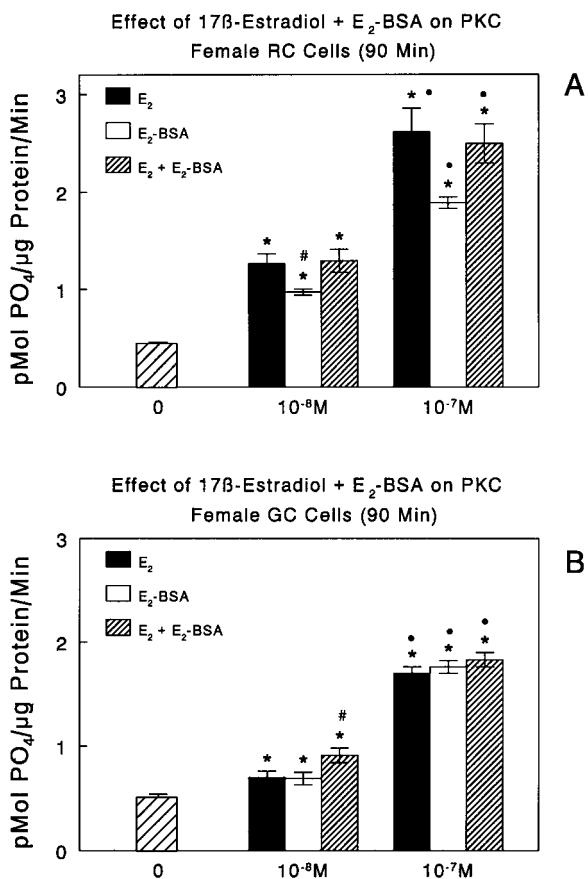Effect of 17β-Estradiol + E₂-BSA on PKC
Female GC Cells (90 Min)

B

Fig. 5. Effect of 17β-estradiol (E₂) and 17β-estradiol-BSA (E₂-BSA) on PKC specific activity. Confluent, fourth passage resting zone (RC, **Panel A**) or growth zone (GC, **Panel B**) chondrocytes from female rats were treated for 90 minutes with control media or media containing $10^{-8}–10^{-7}$ M E₂, E₂-BSA or the two in combination. At harvest, PKC specific activity in the cell layer was determined as described in the Methods. Data represent the mean ± SEM of six cultures from one of two experiments yielding comparable results. *$P < 0.05$, treatment vs vehicle control; #$P < 0.05$, vs E₂ or E₂ + E₂-BSA in Panel A and vs E₂ or E₂-BSA in Panel B; •$P < 0.05$, vs 10^{-8} M.

TABLE I.   Effect of Free E₂ in Media Containing E₂-BSA on PKC Specific Activity of Female Rat Resting Zone Chondrocytes

Treatment	PKC specific activity (pM PO₄/µg protein/min)
Control + vehicle	0.50 ± 0.03
Control filtrate	0.51 ± 0.08
Control retentate	0.46 ± 0.05
10^{-8} M E₂-BSA	2.22 ± 0.11*
10^{-8} M E₂-BSA filtrate	0.62 ± 0.09
10^{-8} M E₂-BSA retentate	2.19 ± 0.12**
10^{-9} M E₂-BSA	1.40 ± 0.06**

Confluent cultures of resting zone chondrocytes from female rats were treated for 90 minutes with control media containing vehicle alone or control vehicle that had been subjected to filtration using a 3000 MW cut off filter. Alternatively, E₂-BSA was filtered and the filtrate or retentate added to the cells. PKC specific activity in the cell layers was determined. Values are the mean ± SEM for N = 6 cultures. Data are from one of two separate experiments, both with comparable results.
*$P < 0.05$, vs control + vehicle medium.
**$P < 0.05$, vs E₂-BSA filtrate.

the PI-PLC inhibitor U73122 had no effect on basal PKC activity, but inhibited PKC activity in E₂-BSA-treated cultures. At 10 µM, U73122 blocked the E₂-BSA effect on PKC completely, whereas the PC-PLC inhibitor D609 had no effect (Fig. 9A). Similarly, in GC cells, U73122 had no effect on basal PKC activity, but 10 µM U73122 blocked the E₂-BSA effect on PKC completely (Fig. 9B). As seen in the RC cultures, D609 had no effect (Fig. 9B).

Role of cyclooxygenase. Prostaglandin production was not necessary for stimulation of PKC by E₂-BSA (Table II). Inhibition of cyclooxygenase caused a dose-dependent increase in basal PKC in resting zone cells as noted

previously [Schwartz et al., 2000]. However, there was no effect on E₂-BSA-stimulated activity. Inhibition of lipooxygenase also had no effect, indicating that leukotriene production was also not required.

Roles of phospholipase D and phosphatidylinositol 3-kinase. PLD was not required for the action of E₂-BSA on PKC in either RC or GC cells (Table III). Wortmannin caused a dose-dependent inhibition of basal PKC in RC cells, but had no effect on basal PKC in GC cells. Similarly, wortmannin had no effect on E₂-BSA-stimulated PKC in either cell type. E₂-BSA had no effect on PLD activity in either RC or GC chondrocytes (data not shown), nor was activity affected by inhibition of PI-PLC with 10 µM U73122 (data not shown). The PI 3-kinase inhibitor LY294022 also had no effect on PKC in either cell type, whether E₂-BSA was present or not (Table IV).

Role of Classic Estrogen Receptor

The effect of E₂-BSA on PKC activity did not involve traditional nuclear estrogen receptors. Neither DES nor ICI 182780 affected basal PKC activity or E₂-BSA-stimulated activity in either GC (Fig. 10) or RC (Fig. 11) cells. When the concentration of ICI 182780 was increased to 10 µM, there was still no effect on PKC activity in control cultures or in cultures treated with 10^{-9} or 10^{-8} M E₂-BSA (data not shown).

Effect of 17ß-Estradiol + GDPßS on PKC

Female RC Cells (90 Min)

Effect of 17ß-Estradiol-BSA + GDPßS on PKC

Female RC Cells (90 Min)

Effect of 17ß-Estradiol + GDPßS on PKC

Female GC Cells (90 Min)

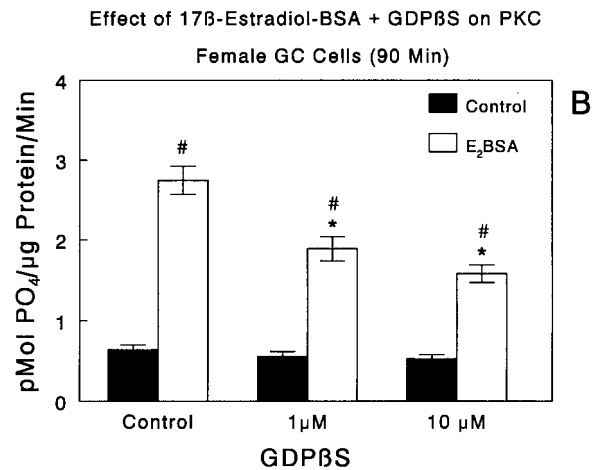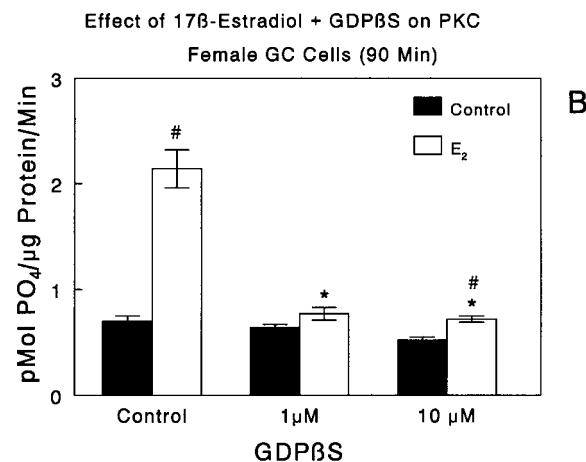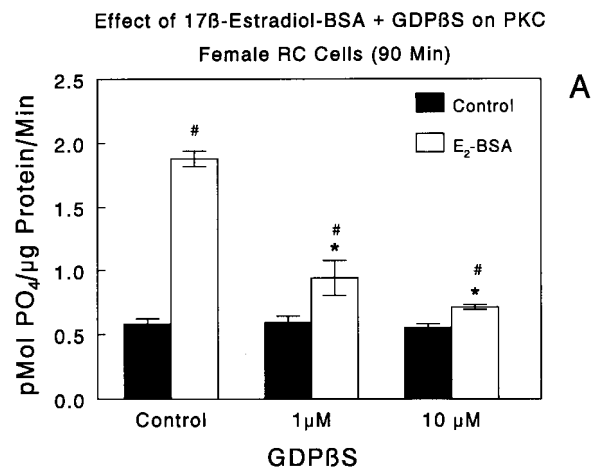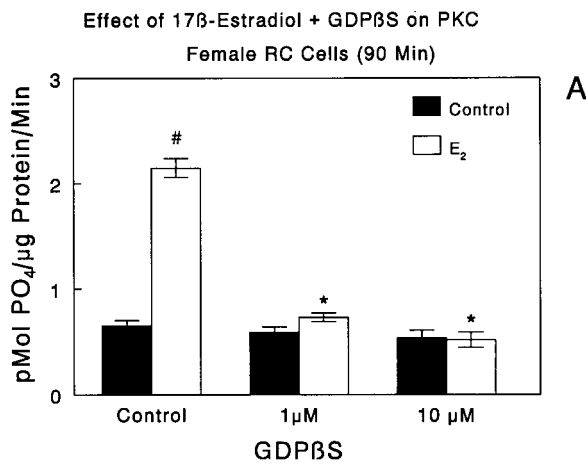Effect of 17ß-Estradiol-BSA + GDPßS on PKC

Female GC Cells (90 Min)

Fig. 6. Effect of the G-protein inhibitor GDPßS on 17β-estradiol-induced PKC specific activity. Confluent, fourth passage resting zone (RC; **Panel A**) or growth zone (GC; **Panel B**) chondrocytes from female rats were treated for 90 minutes with control media or media containing 10^{-8} M E_2 in the presence or absence of 1 or 10 μM GDPßS. At harvest, PKC specific activity in the cell layer was determined as described in the Methods. Data represent the mean ± SEM of six cultures from one of two experiments yielding comparable results. Panel A: *$P < 0.05$, GDPßS vs no GDPßS; #$P < 0.05$, E_2 vs control.

Fig. 7. Effect of the G-protein inhibitor on 17β-estradiol-BSA-induced PKC specific activity. Confluent, fourth passage resting zone (RC, **Panel A**) or growth zone (GC, **Panel B**) chondrocytes from female rats were treated for 90 min with control media or media containing 10^{-8} M E_2-BSA in the presence or absence of 1 or 10 μM GDPßS. At harvest, PKC specific activity in the cell layer was determined as described in the Methods. Data represent the mean ± SEM of six cultures from one of two experiments yielding comparable results. *$P < 0.05$, GDPßS vs no GDPßS; #$P < 0.05$, E_2-BSA vs control.

Similarly, neither 1 μM nor 10 μM ICI 182780 altered the stimulatory effect of medium containing 10^{-9} or 10^{-8} M E_2-BSA that had been filtered to remove any free E_2.

Specificity of Estradiol Effect on PKC Activity

PKC activity of E_2-treated cultures is unaffected by blocking the 1,25-$(OH)_2D_3$ membrane receptor (Fig. 12). Treatment of RC cell cultures with 10^{-8} M 1α,25-$(OH)_2D_3$ had no effect on PKC activity; however, treatment with 10^{-8} M E_2 elicited a significant increase in PKC activity which was unaffected by either Ab99 or non-

specific rabbit IgG (Fig. 12A). Treatment of GC cell cultures with either 10^{-8} M 1α,25-$(OH)_2D_3$ or 10^{-8} M E_2 elicited significant increases in PKC activity. Ab99 blocked only the increase in PKC activity due to 1α,25-$(OH)_2D_3$ and had no effect on PKC activity of E_2-treated cultures (Fig. 12B).

DISCUSSION

A comparison between the effects of E_2-BSA and those of E_2 indicates that many of the effects of E_2 on female rat costochondral chondrocytes

Effect of 17ß-Estradiol-BSA + GTPγS on PKC
Female RC Cells (90 Min)

Effect of 17ß-Estradiol-BSA + PLC Inhibitors on PKC
Female RC Cells (90 Min)

Effect of 17ß-Estradiol-BSA + GTPγS on PKC
Female GC Cells (90 Min)

Effect of 17ß-Estradiol-BSA + PLC Inhibitors on PKC
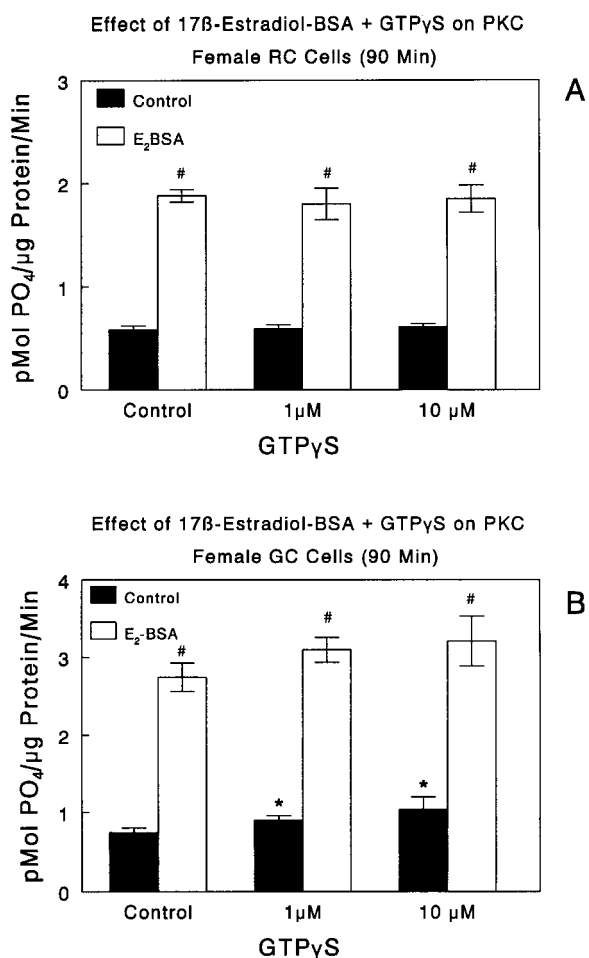Female GC Cells (90 Min)

Fig. 8. Effect of G-protein activation on 17β-estradiol-BSA-induced PKC specific activity. Confluent, fourth passage resting zone (RC, **Panel A**) or growth zone (GC, **Panel B**) chondrocytes from female rats were treated for 90 minutes with control media or media containing 10^{-8} M E_2-BSA in the presence or absence of 1 or 10 μM GTPγS. At harvest, PKC specific activity in the cell layer was determined as described in the Methods. Data represent the mean ± SEM of six cultures from one of two experiments yielding comparable results. *$P < 0.05$, GTPγS vs no GTPγS; #$P < 0.05$, E_2-BSA vs control.
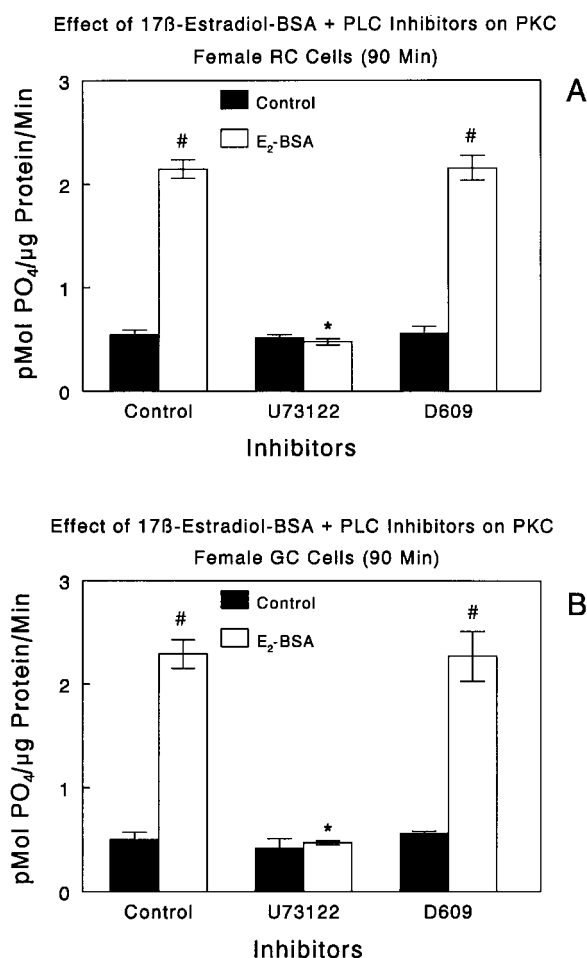
Fig. 9. Effect of PLC inhibitors on 17β-estradiol-BSA-induced PKC specific activity. Confluent, fourth passage resting zone (RC, **Panel A**) or growth zone (GC, **Panel B**) chondrocytes from female rats were treated for 90 minutes with control media or media containing 10^{-8} M E_2-BSA in the absence or presence of 10 μM U73122 or D609. At harvest, PKC specific activity in the cell layer was determined as described in the Methods. Data represent the mean ± SEM of six cultures from one of two experiments yielding comparable results. *$P < 0.05$, U73122 vs no U73122; #$P < 0.05$, E_2-BSA vs control.

involve membrane-mediated mechanisms. E_2-BSA is not hydrolyzed and remains on the outside of the cell [Zheng et al., 1996], yet it mimicked the previously reported effects of E_2 on [^{3}H]-thymidine incorporation, alkaline phosphatase specific activity, and proteoglycan sulfation [Nasatzky et al., 1993, 1994b]. E_2 has been shown to stimulate the PKC signal transduction pathway in RC and GC chondrocytes [Sylvia et al., 1998a] and to mediate its effects on all three of these parameters via this pathway [Sylvia et al., 2000]. The present study shows that E_2-BSA also causes a dose- and time-

dependent increase in PKC specific activity, suggesting that its effects are via PKC-dependent mechanisms as well. Further, at least part of the effect of E_2 may involve receptor-mediated mechanisms that are not membrane-associated.

The effects of E_2-BSA were seen in both RC and GC cells, consistent with our previous observation that both types of cells have receptors for E_2. The methods used in our previous studies, namely E_2 binding to cell homogenates and immunohistochemical detection of E_2 [Nasatzky et al., 1994a], did not discriminate

TABLE II.   Effect of Cyclooxygenase and Lipooxygenase Inhibitors on E_2-BSA-Induced Activation of PKC Specific Activity

Treatment	Protein kinase C specific activity (pM Pi/μg protein/minute)	
	9 min	90 min
Control	0.45 ± 0.02	0.53 ± 0.03
Control $+ 10^{-8}$ M Indomethacin	0.48 ± 0.01	0.56 ± 0.05
Control $+ 10^{-7}$ M Indomethacin	0.54 ± 0.07	$0.68 \pm 0.03*$
Control $+ 10^{-6}$ M Indomethacin	$0.55 \pm 0.03*$	$0.76 \pm 0.03*$
Control $+ 2$ μM NDGA	0.49 ± 0.04	0.48 ± 0.01
Control $+ 20$ μM NDGA	0.49 ± 0.02	0.48 ± 0.01
Control $+ 40$ μM NDGA	0.50 ± 0.04	0.48 ± 0.01
10^{-8} M E_2-BSA	$0.95 \pm 0.05*$	$1.53 \pm 0.06*$
10^{-8} M E_2-BSA $+ 10^{-8}$ M Indomethacin	$0.96 \pm 0.06*$	$1.47 \pm 0.10*$
10^{-8} M E_2-BSA $+ 10^{-7}$ M Indomethacin	$0.94 \pm 0.05*$	$1.49 \pm 0.07*$
10^{-8} M E_2-BSA $+ 10^{-6}$ M Indomethacin	$0.90 \pm 0.04*$	$1.53 \pm 0.08*$
10^{-8} M E_2-BSA $+ 2$ μM NDGA	$0.96 \pm 0.04*$	$1.49 \pm 0.11*$
10^{-8} M E_2-BSA $+ 20$ μM NDGA	$0.96 \pm 0.05*$	$1.43 \pm 0.13*$
10^{-8} M E_2-BSA $+ 40$ μM NDGA	$0.93 \pm 0.02*$	$1.44 \pm 0.08*$

Confluent cultures of resting zone chondrocytes from female rats were treated for 90 minutes $\pm E_2$-BSA. Cyclooxygenase (Cox) activity was inhibited with 10^{-8} to 10^{-6} M indomethacin. Lipooxygenase activity was inhibited with 2, 20, or 40 μM NDGA. Each value is the mean $\pm$ SEM of six cultures. Data are from one of two separate experiments, both yielding comparable results.
$*P < 0.05$, treatment vs control.

between classical nuclear receptors or membrane-associated receptors. Based on immunohistochemical localization of E_2 in the nuclei of the chondrocytes, it is likely that both classical receptors and membrane-associated receptors

TABLE III.   Effect of Phospholipase D Inhibitor on E_2-BSA-Induced Activation of PKC Specific Activity

Treatment	Protein kinase C specific activity (pM Pi/μg protein/minute)	
	RC[a]	GC[a]
Control	0.79 ± 0.07	0.45 ± 0.06
Control $+ 0.1$ μM Wort	0.73 ± 0.05	0.41 ± 0.05
Control $+ 1$ μM Wort	0.69 ± 0.12	0.42 ± 0.07
Control $+ 10$ μM Wort	$0.58 \pm 0.03*$	0.47 ± 0.02
10^{-7} M E_2-BSA	3.78 ± 0.18	1.83 ± 0.10
10^{-7} M E_2-BSA $+ 0.1$ μM Wort	3.92 ± 0.27	1.82 ± 0.12
10^{-7} M E_2-BSA $+ 1$ μM Wort	3.90 ± 0.16	1.77 ± 0.08
10^{-7} M E_2-BSA $+ 10$ μM Wort	3.87 ± 0.14	1.80 ± 0.08

[a]Confluent cultures of female rat costochondral resting zone (RC) and growth zone (GC) chondrocytes were treated for 90 minutes with 10^{-7} M E_2-BSA. Phospholipase D was inhibited with 0.1, 1, or 10 μM wortmannin. Each value is the mean $\pm$ SEM of six independent cultures. Activity of PKC was determined after 90 minutes of treatment. Data are from one of two separate experiments, each yielding comparable results.
$*P < 0.05$, treatment vs control.

exist. However, neither DES nor ICI-182780 affected the stimulation of PKC by E_2-BSA, nor did they alter the effect of E_2 on PKC [Sylvia et al., 2000], indicating that the classical receptors for E_2 do not play a role, at least through 90 min. Moreover, the effect of E_2-BSA was not due to free E_2 present in the medium, since filtrates of the medium containing only free E_2, and not the conjugate, failed to elicit a PKC response. This supports the hypothesis of a membrane receptor-mediated mechanism that does not involve ERα or ERβ directly.

The effects of E_2 and E_2-BSA on RC and GC cells were comparable, but not identical. Resting zone cells were more sensitive to the inhibitory effects of E_2 and E_2-BSA on [3H]-thymidine incorporation than were GC cells. In RC, 10^{-9} M E_2 and E_2-BSA significantly inhibited [3H]-thymidine incorporation, while in GC cells, treatment with 10^{-8} M E_2 was required to see an effect. Further, in GC cells, the inhibitory effect of E_2-BSA was not statistically significant until 10^{-7} M concentrations were used. This suggests that the two cell types may use different mechanisms.

Further evidence that this is the case is the differences in RC and GC response to E_2-BSA with respect to PKC stimulation. E_2-BSA caused an increase in PKC specific activity in RC cells by 3 min, but the effect on GC cells was

**TABLE IV. Effect of Phosphatidylinositol 3-Kinase Inhibitor on
E$_2$-BSA-Induced Activation of PKC Specific Activity**

| Treatment | Protein kinase C specific activity (pM Pi/µg protein /minute) | |
	RC	GC
Control	0.55 ± 0.04	0.50 ± 0.07
Control + 10 µM LY294002	0.59 ± 0.08	0.52 ± 0.07
10^{-7} M E$_2$-BSA	$2.15 \pm 0.09*$	$2.29 \pm 0.14*$
10^{-7} M E$_2$-BSA + 10 µM LY294002	$2.19 \pm 0.09*$	$2.21 \pm 0.18*$

Confluent cultures of female rat costochondral resting zone (RC) and growth
zone (GC) chondrocytes were treated for 90 minutes with E$_2$-BSA. Phospha-
tidylinositol-3-kinase activity was inhibited with 10 µM LY294002. Each value
is the mean $\pm$ SEM of six independent cultures from one of two separate
experiments, both yielding comparable results.
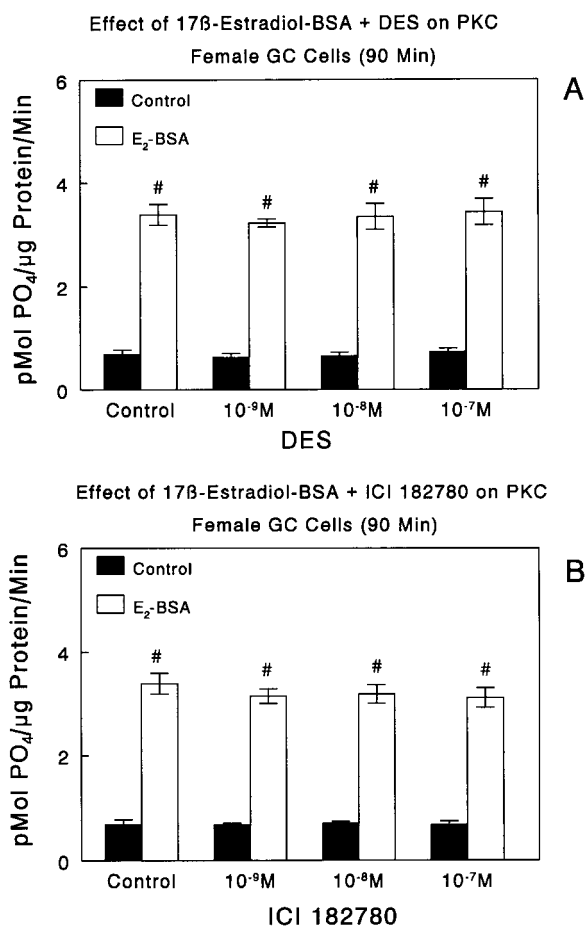*$P < 0.05$, treatment vs control.

Fig. 10. Effect of 17β-estradiol receptor agonist diethylstilbes-
terol (DES) and antagonist ICI 182780 on PKC specific activity.
Confluent, fourth passage growth zone (GC) chondrocytes from
female rats were treated for 90 minutes with control media or
media containing 10^{-8} M E$_2$-BSA in the presence or absence of
10^{-9}–10^{-7} M DES (**Panel A**) or ICI 182780 (**Panel B**). At harvest,
PKC specific activity in the cell layer was determined as
described in the Methods. Data represent the mean $\pm$ SEM of six
cultures from one of two experiments yielding comparable
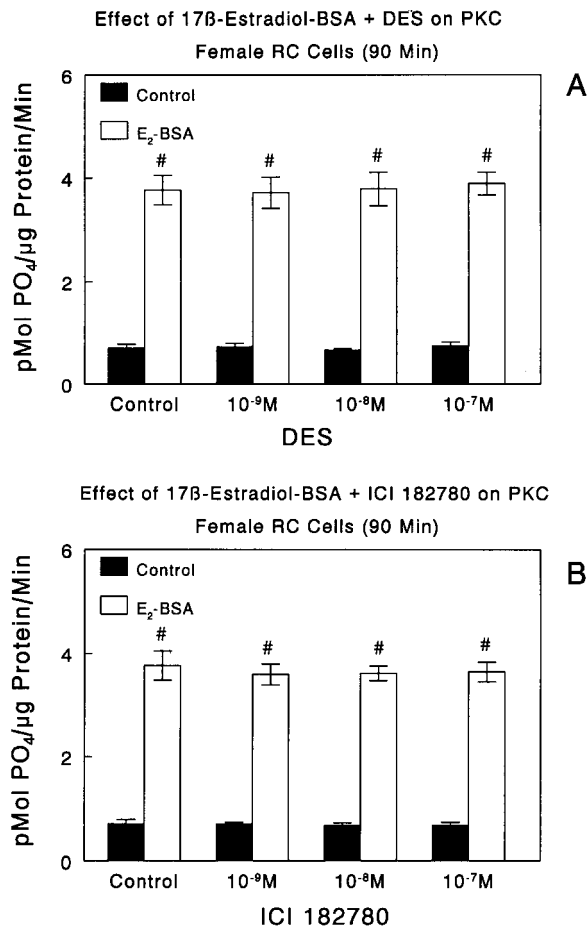results. [#]$P < 0.05$, E$_2$-BSA vs control.

Fig. 11. Effect of 17β-estradiol receptor agonist diethylstilbes-
terol (DES) and antagonist ICI 182780 on PKC specific activity.
Confluent, fourth passage resting zone (RC) chondrocytes from
female rats were treated for 90 minutes with control media or
media containing 10^{-8} M E$_2$-BSA in the presence or absence of
10^{-9}–10^{-7} M DES (**Panel A**) or ICI 182780 (**Panel B**). At harvest,
PKC specific activity in the cell layer was determined as
described in the Methods. Data represent the mean $\pm$ SEM of six
cultures from one of two experiments yielding comparable
results. *$P < 0.05$, E$_2$-BSA vs control.

Specificity of E_2 Effect on PKC Female RC Cells (90 Min)

A

Specificity of E_2 Effect on PKC Female GC Cells (9 Min)
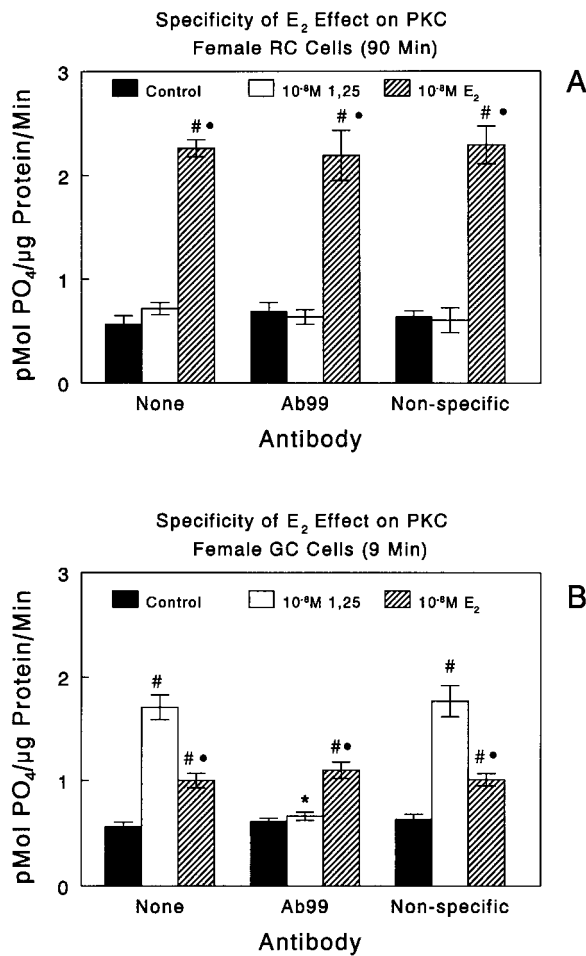

B

Fig. 12. Specificity of 17β-estradiol (E_2) effect on PKC specific activity. Confluent, fourth passage resting zone (RC) (**Panel A**) and growth zone (GC) (**Panel B**) chondrocytes were treated for 90 or 9 minutes, respectively, with control media, 10^{-8} M 1,25-$(OH)_2D_3$, or 10^{-8} M E_2 in the absence or presence of 1/500 dilutions of Ab99 or rabbit immunoglobulin G2. At harvest, PKC specific activity in the cell layer was determined as described in the Methods. Data represent the mean ± SEM of six cultures from one of two experiments yielding comparable results. *$P < 0.05$, vs none and non-specific IgG2; #$P < 0.05$, treatment vs control; •$P < 0.05$, vs 1,25-$(OH)_2D_3$.

evident within 1 min. Peak activity in GC cells also occurred sooner, and the rate of increase was more rapid. The effects of E_2-BSA and E_2 on PKC were identical, and no difference in activity was noted when GC cells were exposed to the two forms of estradiol together. In contrast, E_2 elicited a greater increase in PKC in RC cells than was seen in response to E_2-BSA, and when the two forms of estradiol were used together, the effect of E_2 predominated. This suggests that PKC activity in RC cells involves pathways activated at the cytoplasmic mem-

brane as well as intracellularly, whereas PKC activity in GC cells is maximally activated by membrane-associated mechanisms.

As noted previously for E_2 [Sylvia et al., 1998a], the time course of action in both RC and GC cells suggests that multiple mechanisms may be involved in the activation of PKC by E_2-BSA. Activity continued to increase in both cell types, at least up to 90 min. Log phase increases occurred in RC cells through 90 min and in GC cells between 9 and 30 min. This suggests a rapid initial effect followed by a delayed response. Since PKC activation by E_2 is largely independent of gene transcription or protein synthesis, at least over the first 90 min [Sylvia et al., 1998a], it is likely that E_2 and E_2-BSA initiate one or more signal transduction cascades that amplify the PKC response [Sylvia et al., 2000]. Underlying differences in the phenotypic expression of the two populations of chondrocytes, including differences in membrane phospholipid composition and metabolism and handling of Ca^{2+} ions [Boyan et al., 1997], may contribute to the differences in time course as discussed below.

The PKC isoform sensitive to E_2 is PKCα [Sylvia et al., 1998a], and this isoform of PKC is sensitive to both Ca^{2+} ions and phospholipids [Ohno et al., 1991; Hug and Sarre, 1993]. We did not determine whether E_2-BSA also exerts its effects through PKCα, but the assay conditions that we used were optimized for this isoform [Bell et al., 1986]. Rapid changes in Ca^{2+} ion flux could contribute to changes in activity noted in the present study, since rapid movement of Ca^{2+} ions in response to E_2 has been observed in a number of cell types [Wehling, 1997; Mermelstein et al., 1996; Dayanithi and Tapia-Arancibia, 1996]. Recent studies have also shown that high doses of E_2 (>1 μM) result in acute nongenomic activation of maxi-K channels via binding to the β-subunit [Valverde et al., 1999]. It is unlikely that such a mechanism is involved in the present study, however, since E_2 and E_2-BSA concentrations of 0.1 to 100 nM elicit the PKC response.

Phospholipid metabolism is involved in the stimulation of PKC by both E_2 and E_2-BSA in the chondrocytes. Both forms of estradiol require PI-PLC rather than PC-PLC. Moreover, the PLC involved is coupled to a G-protein that is sensitive to inhibition by GDPβS. Previously, we reported that the action of E_2 on chondrocytes is pertussis toxin-insensitive and cholera

toxin-insensitive, suggesting that the G-proteins involved are not Gi and Gs, respectively [Sylvia et al., 2000]. One possibility is PLCβ, which is coupled to the pertussis toxin-insensitive Gq [Taylor and Exton, 1991].

While PLC is involved in the mechanism of both E_2 or E_2-BSA action, PLD is not. Neither form of estradiol had an effect on PLD activity in either RC or GC cells. Similarly, inhibition of PLD with wortmannin had no effect on E_2 or E_2-BSA-stimulation of PKC activity in either cell type. Moreover, specific inhibition of PI 3-kinase also had no effect on PKC activity in response to E_2-BSA, indicating that the action of wortmannin was specific to PLD [Carrasco-Marin et al., 1994] and demonstrating that PI 3-kinase does not play a role in the regulation of PKC by E_2-BSA. Specific inhibition of PI-PLC had no effect on PLD activity, whether cells were stimulated with E_2-BSA or not, indicating that an upstream increase in PKC was not necessary to exert an increase in PLD, as has been noted in other systems [Exton, 1997]. Thus, PLD does not play a role in the rapid activation of PKC or in the amplification of the E_2-BSA-dependent increase in PKC activity.

E_2 has been shown to cause a rapid increase in arachidonic acid turnover in costochondral chondrocytes [Schwartz et al., 1996], indicating that it regulates both deacylation and reacylation of arachidonic acid. While it is possible that the arachidonic acid might play a direct role in modulating E_2 and E_2-BSA activity since this fatty acid can act through its own nuclear receptors [Bocos et al., 1995], the results of the present study indicate that further metabolism of arachidonic acid to prostaglandin or leukotriene is not involved in the mechanism. Although inhibition of prostaglandin production by indomethacin resulted in an increase in basal PKC in RC cells [Helm et al., 1996], neither the general Cox inhibitor, nor the lipoxygenase inhibitor NDGA, altered the stimulatory effect of either form of estradiol on PKC activity in either RC or GC cells. The possibility remains that E_2 and E_2-BSA exert their effects through another phospholipase A_2-dependent signaling mechanism, however.

The recognition that E_2 exerts some of its actions via rapid membrane-mediated mechanisms is relative new [Fiorelli et al., 1996; Mermelstein et al., 1996; Valverde et al., 1999]. The data presented here support the hypothesis that direct membrane effects are a general property of steroid and secosteroid hormones. However, it is clear that E_2 elicits a hormone-specific response as well. The membrane-associated effects of E_2 and E_2-BSA described here are distinct from those previously reported for either $1\alpha,25$-$(OH)_2D_3$ or $24R,25$-$(OH)_2D_3$, [Schwartz and Boyan, 1988; Schwartz et al., 1990; Swain et al., 1993; Helm et al., 1996; Sylvia et al., 1998b, 1993]. Moreover, even though PI-PLC is required for stimulation of PKC by either $1\alpha,25$-$(OH)_2D_3$ [Sylvia et al., 1993] or E_2 and E_2-BSA, neither of the estradiols elicited their effects on PKC through the same receptor as used by $1\alpha,25$-$(OH)_2D_3$. E_2 and E_2-BSA both stimulated PKC activity in RC cells, which do not respond to $1\alpha,25$-$(OH)_2D_3$ with an increase in PKC [Sylvia et al., 1993]. In addition, antibody 99, which is specific to the $1\alpha,25$-$(OH)_2D_3$ membrane receptor (1,25-mVDR) [Nemere et al., 1998], failed to block the effects of either E_2 or E_2-BSA on PKC activity in GC cells, although it effectively blocked the effects of $1\alpha,25$-$(OH)_2D_3$ on this enzyme activity.

Although E_2 and E_2-BSA share a common mechanism for activating PKC activity in RC and GC cells, there are some differences as well. While E_2 can enter the cell, E_2-BSA cannot [Zheng et al., 1996]. Thus, the effects of E_2 may involve mechanisms in addition to the membrane-mediated mechanisms elicited by E_2-BSA. This appears to be the case in the chondrocytes. The effect of E_2 on PKC activity in RC cells was more robust than the effect of E_2-BSA, suggesting that in these cells at least, E_2 elicited both a membrane-mediated response and an intracellular response. This interpretation is supported by the observation that the G-protein inhibitor GDPβS completely blocked the effect of E_2 on PKC activity in both RC and GC cells, but it only partially inhibited the effect of E_2-BSA. Since GDPβS can exert its effects intracellularly [Gilman, 1987], this suggests that E_2-BSA initiates a cascade by direct action on a cell surface receptor, whereas E_2, once internalized, may act through mechanisms that are more sensitive to the action of G-proteins. The initial activation of the cascade at the membrane is important, though, because intracellular activation of G-proteins with GTPγS had no effect on either E_2 or E_2-BSA-stimulated PKC activity.

When GC chondrocytes are treated with E_2 and E_2-BSA in combination, the resultant PKC

activity is identical to that seen in cultures treated with either hormone alone, suggesting that they share a common receptor. Similarly, the effects of E_2 and E_2-BSA together on PKC activity in RC cells are comparable to that of E_2 alone. This common receptor is membrane-associated, since the cell membrane is impermeable to this conjugated form of the hormone [Zheng et al., 1996]. It is currently unknown whether chondrocytes express a membrane receptor for E_2; however, putative E_2 membrane receptors have been reported in other systems [Fiorelli et al., 1996; Ramirez et al., 1996; Benten et al., 1998].

The E_2/E_2-BSA membrane receptor is unlikely to be a membrane-associated form of the traditional estrogen receptor ERα [Razandi et al., 1999] or ERβ. In contrast to E_2, E_2-BSA does not bind to either ERα or ERβ, as assessed by electrophoretic mobility shift assays [Stevis et al., 1999]. It is certainly possible that E_2 exerts some of its effects on PKC activity through intracellular estrogen receptor-mediated mechanisms, but here, too, it is unlikely to be ERα or ERβ. Neither the estrogen receptor agonist DES, nor the antagonist ICI 182780 [Wakeling, 1995], had an effect on PKC, and neither compound affected PKC stimulated by E_2 or E_2-BSA. Other signaling mechanisms are likely to be involved. E_2 has been shown to generate rapid Ca^{2+} ion flux [Lieberherr et al., 1989], which can have downstream effects on a number of signaling pathways, including PKC activation [Sylvia et al., 2000], resulting in activation of MAP kinase [Endoh et al., 1997], and ultimately in new gene expression. Similarly, E_2-BSA has been shown to elicit nongenomic actions in other systems, such as elevation of cytosolic calcium in T lymphocytes [Benten et al., 1998], nitric oxide release by monocytes [Stefano et al., 1999], and MAP kinase activation in neuroblastoma cells [Stevis et al., 1999]. All of these intracellular mediators can modulate gene transcription through nonERα- or ERβ-dependent mechanisms.

ACKNOWLEDGMENTS

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