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The membrane effects of 17β-estradiol on chondrocyte phenotypic expression are mediated by activation of protein kinase C through phospholipase C and G-proteins

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Abstract

Growth plate chondrocytes from both male and female rats have nuclear receptors for 17β-estradiol (E_2); however, recent studies indicate that an alternative pathway involving a membrane receptor may also be involved in the female cell response. E_2 directly affects the fluidity of chondrocyte membranes derived from female, but not male, rats. In addition, E_2 activates PKC in a nongenomic manner in female cells, and chelerythrine, a specific inhibitor of PKC, inhibits E_2-dependent alkaline phosphatase activity in these cells, indicating PKC is involved in the signal transduction mechanism. The aims of this study were: (1) to examine if PKC mediates the effect of E_2 on chondrocyte proliferation, differentiation, and matrix synthesis; and (2) to determine the pathway that mediates the membrane effect of E_2 on PKC. Confluent, fourth passage resting zone (RC) and growth zone (GC) chondrocytes from female rat costochondral cartilage were treated with 10^{-10} to 10^{-7} M E_2 in the presence or absence of the PKC inhibitor chelerythrine, and changes in alkaline phosphatase specific activity, proteoglycan sulfation, and [^{3}H]thymidine incorporation were measured. To examine the pathway of PKC activation, chondrocyte cultures were treated with E_2 in the presence or absence of genistein (an inhibitor of tyrosine kinases), U73122 or D609 (inhibitors of phospholipase C [PLC]), quinacrine (an inhibitor of phospholipase A_2 [PLA_2]), and melittin (an activator of PLA_2). Alkaline phosphatase specific activity and proteoglycan sulfation were increased and [^{3}H]thymidine incorporation was decreased by E_2. The effects of E_2 on all parameters were blocked by chelerythrine. Treatment of the cultures with E_2 produced a significant dose-dependent increase in PKC. U73122 dose-dependently inhibited the activation of PKC in E_2-stimulated female chondrocyte cultures. However, the classical receptor antagonist ICI 182780 was unable to block the stimulatory effect of E_2 on PKC. Moreover, the classical receptor agonist diethylstilbestrol (DES) had no effect on PKC, nor did it alter the stimulatory effect of E_2. Inhibition of tyrosine kinase and PLA_2 had no effect on the activation of PKC by E_2. The PLA_2 activator also had no effect on PKC activation by E_2. E_2 stimulated PKC activity in membranes isolated from the chondrocytes, demonstrating a direct membrane effect for this steroid hormone. These data indicate that the rapid nongenomic effect of E_2 on PKC activity in chondrocytes from female rats is sex-specific and dependent upon a G-protein-coupled phospholipase C.

Keywords: Chondrocyte cultures; 17β-Estradiol; Protein kinase C; Signal transduction; Phospholipase C; G-proteins

1. Introduction

17β-Estradiol (E_2) regulates endochondral bone formation directly through action on chondrocytes and indirectly through secretion of other hormones and local factors [1–4]. E_2 mediates its effects on cells through steroid hormone receptors [5], the classical

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estrogen receptor, now referred to as estrogen receptor-α, and a second receptor called estrogen receptor-β [6]. Specific receptors for E_2 have been found in articular cartilage [7], growth plate cartilage [8,9], and fracture callus [10].

Some of the biological effects of E_2 are mediated by rapid, membrane-associated mechanisms in various cell types [11–15]. In bone, rapid effects of E_2 have been linked to changes in calcium flux in osteoblasts [16] and acid production by osteoclasts [17]. We have shown that some of the effects of E_2 on growth plate chondrocytes are mediated by rapid, membrane-associated mechanisms [18,19]. Using rat costochondral chondrocytes as a model for studying agents which modulate cartilage cell differentiation and maturation [20], we have shown that E_2 causes an increase in arachidonic acid turnover, phospholipase A_2 activity, and membrane fluidity in cultures of chondrocytes derived from female rats [18]. Additionally, E_2 stimulates protein kinase C (PKC) activity in a dose-dependent manner via mechanisms that are independent of new gene expression [19].

These rapid events may be due to a unique membrane receptor for the hormone that is structurally distinct from the nuclear receptor [21,22]. Our studies support this hypothesis. We have shown that E_2 affects fluidity of matrix vesicles isolated from female, but not male, chondrocyte cultures. Since these extracellular organelles contain no DNA or protein synthesis capabilities, 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.

Steroid hormones can function via pathways traditionally ascribed to peptide hormones, including PKC [23–26] and MAP kinase [27]. PKC is a family of serine/threonine kinases that transduce signals from extracellular growth factors and hormones to intracellular effector molecules [28]. Other investigators have suggested a role for PKC in the biological response to E_2 by demonstrating that PKC inhibitors block the action of this steroid on DNA synthesis [29,30]. In rat costochondral chondrocyte cultures, the lipophilic secosteroids, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, stimulate PKC activity in a cell maturation-dependent manner, with 1,25-$(OH)_2D_3$ regulating the enzyme activity in growth zone cells and 24,25-$(OH)_2D_3$ regulating the enzyme activity in resting zone cells [31]. Both genomic and nongenomic mechanisms are involved in the process, including differential segregation of PKC isoforms into plasma membrane and matrix vesicle compartments and effects of the vitamin D metabolites on PKC, which are specific to the membrane fraction examined [32]. Recent studies have demonstrated that specific membrane receptors exist for each metabolite [33,34].

17β-Estradiol is a steroid hormone, whereas vitamin D metabolites are seco-steroids; thus, they are likely to exert similar, but not identical, effects on membrane signaling pathways such as PKC. In addition, chondrocyte response to E_2 is gender-dependent [3,35–38], which is not the case for the vitamin D metabolites (unpublished data). The aims of the present study were to examine the role of PKC in E_2 regulation of chondrocyte proliferation, differentiation, and matrix synthesis and to determine the pathway mediating the membranous effect of E_2 on PKC. We examined the role of PKC by testing the effects of PKC inhibition on [^{3}H]thymidine incorporation, alkaline phosphatase specific activity, and proteoglycan sulfation in E_2-treated resting zone and growth zone chondrocyte cultures. To determine the pathway mediating the effect of E_2, we examined the effects of phospholipase C, phospholipase A_2, cyclooxygenase (Cox) and lipoxygenase inhibition, as well as the action of estrogen receptor agonists and antagonists on PKC activity in E_2-treated cultures.

2. Materials and methods

2.1. Reagents

17β-estradiol, 17α-estradiol, diethylstilbesterol (DES), G-protein inhibitors (pertussis toxin, cholera toxin, and GDPβS) [39], indomethacin, and quinacrine (phospholipase A_2 inhibitor) [40] were purchased from Sigma Chemical Co. (St Louis, MO). The estrogen receptor antagonist ICI 182780 [41] was obtained from Tocris Cookson Inc. (Ballwin, MO). The following chemicals were purchased from Calbiochem (San Diego, CA): 1,2-dioctanoyl-*sn*-glycerol (DOG), chelerythrine (PKC inhibitor) [42], genistein (tyrosine kinase inhibitor) [43], U73122 (phospholipase C inhibitor) [44], and D609 (phosphatidylcholine-specific phospholipase C inhibitor) [45]. The COX-1 specific inhibitor resveratrol [46] was obtained from Cayman Chemical (Ann Arbor, MI). Phospholipase A_2 activators mastoparan [47] and melittin [48] and the lipoxygenase inhibitor nordihydroguaiaretic acid [49] were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). PKC assay reagents and DMEM were obtained from GIBCO-BRL (Gaithersburg, MD). The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay reagent [50] obtained from Pierce Chemical Co. (Rockford, IL). [^{32}P]ATP, [^{3}H]thymidine and [^{35}S]sulfate were obtained from NEN-DuPont (Boston, MA).

2.2. Chondrocyte cultures

The culture system used in this study has been described in detail previously [51]. Chondrocytes were

isolated from the resting zone (RC; reserve zone) and growth zone (GC; prehypertrophic/upper hypertrophic cell zones) of the costochondral junction of 125 g female Sprague–Dawley rats by enzymatic digestion and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) [18,19] 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. 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 [52]. Furthermore, they retain their differential responsiveness to vitamin D metabolites at this passage [51,53], as well as differential responsiveness to a number of other factors [54].

Since normal serum estradiol ranges from 10^{-9} to 10^{-8} M, we chose to test E_2 at physiological and pharmacological concentrations ranging from 10^{-10} to 10^{-7} M. In some parallel experiments, the inactive stereoisomer, 17α-estradiol (17α) was used at similar concentrations as a control. Both E_2 and 17α were dissolved in absolute ethanol and diluted in culture medium by at least 1000-fold to the required concentration. Control cultures contained ethanol at the highest concentration used in the experimental cultures.

2.3. Role of PKC in mediating the physiologic response to E_2

2.3.1. [^{3}H]thymidine incorporation

E_2 inhibits [^{3}H]thymidine incorporation by RC and GC chondrocytes [35]. To determine if this response to E_2 is mediated by PKC, DNA synthesis was estimated by measuring [^{3}H]thymidine incorporation into trichloroacetic acid (TCA) insoluble cell precipitates as described previously [53]. Quiescence was induced by incubating confluent cultures for 48 h in DMEM containing 1% FBS. The medium was then replaced with DMEM containing 1% FBS alone (control), 10^{-10}–10^{-7} M E_2 or E_2 plus 10 μM chelerythrine (PKC inhibitor) for 24 h. Two hours prior to harvest, [^{3}H]thymidine was added.

2.3.2. Alkaline phosphatase specific activity

E_2 has been shown previously to stimulate alkaline phosphatase specific activity in both RC and GC chondrocyte cultures [35]. To determine if this effect is mediated by PKC, confluent cells were treated with medium containing vehicle alone, 10^{-10}–10^{-7} M E_2 or E_2 plus 10 μM chelerythrine for 24 h. 17α-estradiol was used at similar concentrations in a control experiment. 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 [55,56].

2.3.3. Proteoglycan sulfation

E_2 stimulates proteoglycan sulfation in RC and GC chondrocyte cultures [36]. To determine if this is mediated by PKC, proteoglycan synthesis was assessed by measuring [^{35}S]sulfate incorporation by confluent cultures as described previously [36,57]. At confluence, fresh medium containing vehicle alone, 10^{-10}–10^{-7} M E_2 or E_2 plus 10 μM chelerythrine was added for an additional 24 h. Four hours prior to harvest, 50 μl DMEM containing 18 μCi/ml [^{35}S]sulfate and 0.814 mM carrier sulfate were added to each culture. 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 [50] in the cell layer.

2.4. Mechanism of E_2-dependent PKC activation

To determine the signaling pathways involved in E_2-dependent activation of PKC, 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 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 [58], experimental time points of 9, 90 and 270 min were chosen. After the appropriate incubation period, cell layers were washed with phosphate buffered saline (PBS), loosened from the wells with a sterile cell scraper, 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 [50] and PKC activity [31].

2.5. Protein kinase C assay

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 [59]. To this mixture, a high affinity myelin basic protein peptide substrate and [^{32}P]ATP (25 μCi/ml) was added to a final volume of 50 μl. Following a 10-min incubation at 30°C, samples were spotted onto phosphocellulose discs, washed, and counted in a scintillation counter.

2.5.1. Role of classical estrogen receptor

To assess the role of the classical estrogen receptor in the effect of E_2 on PKC, female RC cells were treated with E_2 in the presence or absence of estrogen receptor agonist DES or antagonist ICI 182780. RC cultures were treated for 90 min 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.

2.5.2. Phospholipase C

Phospholipase C (PLC) involvement in E_2 action was assessed using U73122, an inhibitor of phosphatidylinositol (PI)-specific PLC [44], and D609, an inhibitor of phosphatidylcholine (PC)-specific PLC [45]. Cultures were incubated for 90 min in media containing 10^{-9} M–10^{-8} M E_2 plus 0, 1 or 10 μM U73122 or with 1, 10 or 100 μM D609. Controls contained the E_2 and U73122 vehicles, ethanol and PBS. To examine the effect of PLC inhibition over time, cultures were treated with control media, 10^{-8} M E_2, 10 μM U73122, or E_2 + U73122 for 9, 90 or 270 min. To further assess the involvement of PLC we examined the effect of diacylglycerol, the product of PLC action [60]. Cultures were treated for 90 min with vehicle alone, or 10^{-9}–10^{-8} M E_2 in the presence or absence of 1, 10 or 100 μM dioctanoylglycerol (DOG).

2.5.3. G-proteins

To assess the role of G-proteins, pertussis toxin (PTX, Gi inhibitor), cholera toxin (CTX, Gs inhibitor), and the nonhydrolyzable GDPβS (general G-protein inhibitor) were used. Cultures were treated for 90 min with control media or 10^{-7} M E_2 in the presence or absence of 1, 10, or 100 ng/ml PTX or CTX. In a parallel set of experiments, cultures were treated for 90 min with control media, 10^{-8} M E_2, or E_2 + 100 ng/ml PTX in the presence or absence of 1, 10 or 100 μM GDPβS. To examine the effect of G-protein inhibition over time, cultures were treated with control media, 10^{-8} M E_2, 100 μM GDPβS, or E_2 + GDPβS for 9, 90, or 270 min.

2.5.4. Tyrosine kinases

To assess the role of tyrosine kinases, cultures were incubated with control media, 10^{-8} M E_2, 10 μM genistein [43] or E_2 + genistein for 9, 90, or 270 min. Control cultures contained the E_2 vehicle (ethanol) and the genistein vehicle (PBS).

2.5.5. Phospholipase A_2

To examine the role of phospholipase A_2 (PLA_2), melittin [48] or mastoparan [47] were used to activate PLA_2 activity. Quinacrine [40] was used to inhibit PLA_2 activity. Cultures were treated with control media, 10^{-8} M E_2, 3 μg/ml melittin, or E_2 + melittin for 9, 90, or 270 min and assayed for PKC activity. Melittin or quinacrine were tested in a similar manner at final concentrations of 20 μg/ml or 10μM, respectively.

2.5.6. Cyclooxygenase and lipoxygenase

To examine the role of cyclooxygenase (COX) and lipoxygenase (LPX), the general COX inhibitor indomethacin [61] and the LPX inhibitor nordihydroguaiaretic acid (NDGA) [49] were used. Cultures were treated for 90 min with control media or 10^{-8} M E_2 in the presence or absence of 10^{-8}–10^{-6} M indomethacin. NDGA was tested in a similar manner at final concentrations of 2, 20, or 40 μM.

2.6. Direct effect of 17β-estradiol on membrane fractions

To verify that the effect of E_2 on PKC activity is nongenomic, matrix vesicles and plasma membranes were isolated from confluent, fourth passage RC cultures from female rats as described previously [20,56,62] and incubated directly with the hormone [32,62]. For each experiment, there was an N of six membrane preparations for each variable. Each membrane preparation was derived from the pooled membranes from two separate cultures (i.e., two T-75 flasks). Matrix vesicles or plasma membranes (30 μl of 1 mg/ml preparations in 0.9% NaCl containing 10% FBS) were incubated in the absence (vehicle only) or presence of a final concentration of 10^{-9}–10^{-7} M E_2 for either 3, 9, 90, or 270 min at 37°C. Following incubation, samples were assayed for PKC activity.

2.7. Statistical management of data

For each experiment, each data point represents the mean ± SEM for six individual cultures (cell layers) or six membrane samples. Significance between groups was determined by Bonferroni's modification of the 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.

3. Results

3.1. The role of PKC in the physiological response to E_2

3.1.1. [^{3}H]thymidine incorporation

DNA synthesis was regulated in a PKC-dependent manner in both RC and GC cells from female rats (Fig. 1). Chelerythrine inhibited DNA synthesis in control cultures of both cell types. In E_2-treated RC cultures, the inhibitory effect of chelerythrine was even greater

than that of E_2. This was also the case for GC cells, but only at the lowest E_2 concentration examined.

3.1.2. Alkaline phosphatase specific activity

E_2 regulated alkaline phosphatase specific activity in RC cells via PKC. Chelerythrine reduced E_2-stimulated activity in a dose-dependent manner (Fig. 2, top panel). At E_2 concentrations $\leq 10^{-9}$ M, however, 10 μM chelerythrine was unable to block the E_2-stimulated enzyme activity completely. Chelerythrine caused a small decrease in basal PKC activity (bottom panel), as has been noted previously [63]. The effect of E_2 was specific, since 17α-estradiol, the inactive isomer, had no effect on alkaline phosphatase in these cells.

PKC also mediated the response of GC cells to E_2 (Fig. 3). Chelerythrine blocked the effect of E_2, regardless of the concentration of E_2 used. In contrast to RC cultures, chelerythrine did not inhibit basal enzyme activity.

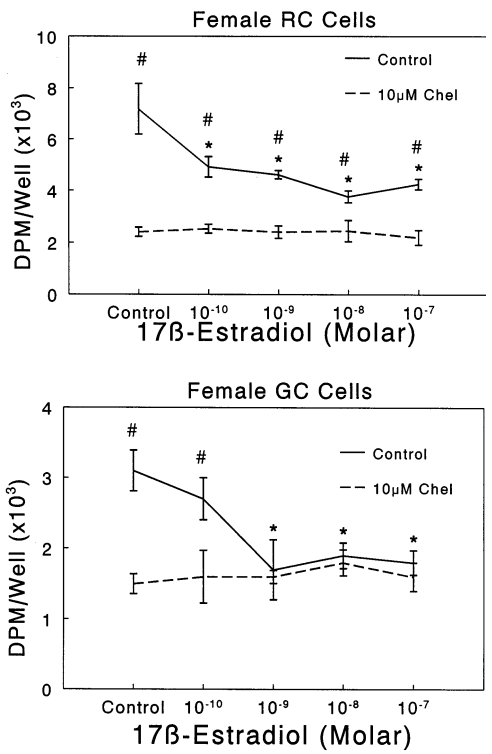

Fig. 1. Effect of the PKC inhibitor chelerythrine on 17β-estradiol-(E_2) induced [^{3}H]thymidine incorporation by resting zone and growth zone chondrocytes. Confluent, fourth passage resting zone (RC, upper panel) or growth zone (GC, lower panel) cells from female rats were treated for 24 h with control media or media containing 10^{-10}–10^{-7} M E_2 in the presence or absence of 10 μM chelerythrine. Two hours prior to harvest, [^{3}H]thymidine was added to the cultures. At harvest, the cell layers were washed, precipitated with trichloroacetic acid as described in Section 2, and counted in a scintillation counter. Values are the mean ± SEM of six cultures from one of three experiments, all with comparable results. $^*P < 0.05$, E_2 treatment vs. control; $^{\#}P < 0.05$, Chel vs. control.

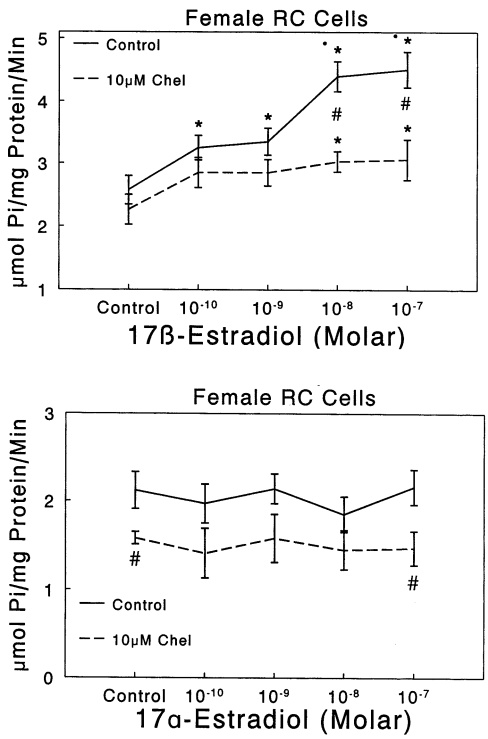

Fig. 2. Effect of the PKC inhibitor chelerythrine on 17β-estradiol-(E_2) induced alkaline phosphatase specific activity of resting zone chondrocytes. Confluent, fourth passage resting zone cells (RC) from female rats were treated for 24 h with control media or media containing 10^{-10}–10^{-7} M E_2 (upper panel) or 10^{-10}–10^{-7} M 17α-estradiol (lower panel) in the presence or absence of 10 μM chelerythrine. At harvest, alkaline phosphatase specific activity in the cell layer was determined as described in Section 2. Each data point is the mean ± SEM of six cultures from one of three experiments, all with comparable results. $^*p < 0.05$, E_2 treatment vs control; $^{\#}P < 0.05$, Chel vs. control; $^{\bullet}P < 0.05$, 10^{-8}–10^{-7} M E_2 vs. 10^{-10}–10^{-9} M E_2.

3.1.3. Proteoglycan sulfation

As expected [36], E_2 in chondrocytes increased proteoglycan sulfation in female GC cells (Fig. 4). The effect of E_2 was blocked by PKC inhibition. Similar data were obtained for female RC cultures (data not shown).

3.2. Mechanism of E_2-dependent PKC activation

3.2.1. Role of classic estrogen receptor

The activation of PKC by E_2 in chondrocytes does not involve classical nuclear E_2 receptors (Table 1). PKC activity was not significantly changed in RC cultures treated with either 10^{-9}–10^{-7} M diethylstilbesterol (DES) or ICI 182780. Further, neither compound affected basal PKC activity or E_2-stimulated activity.

3.2.2. Role of phospholipase C

The effect of E_2 on PKC involved the action of phospholipase C in RC cells (Fig. 5). The PI-PLC

inhibitor U73122 had no effect on basal PKC activity, but inhibited PKC activity in E_2-treated RC cultures in a dose-dependent manner (Fig. 5A). At 10 μM, U73122 blocked the E_2 effect on PKC completely at all time points tested (Fig. 5B). In contrast, the PC-PLC inhibitor D609 was without effect (Table 2). In GC cultures, as in RC cultures, the PI-PLC inhibitor U73122 blocked E_2-induced PKC activity (Fig. 6). PKC activity in control cultures was unaffected, but a dose-dependent decrease in PKC activity was observed in both 10^{-9} M and 10^{-8} M E_2 in cultures treated for 90 min (Fig. 6A). As noted for RC cultures, at 9 min, 10 μM U73122 reduced PKC in E_2-treated cultures to levels below those seen in control cultures (Fig. 6B).

Dioctanoylglycerol (DOG) significantly increased PKC activity in control and E_2-treated cultures of RC cells in a dose- and time-dependent manner (Fig. 7). RC cultures treated for 90 min with 10^{-9}–10^{-8} M E_2 in the presence of 10 or 100 μM DOG had higher PKC activity than cultures receiving E_2 alone, and the effects of DOG and E_2 were additive (Fig. 7A). The additive effects of DOG and E_2 were also noted at 9 and 270 min (Fig. 7B). Similar results were seen for GC cells (data not shown).

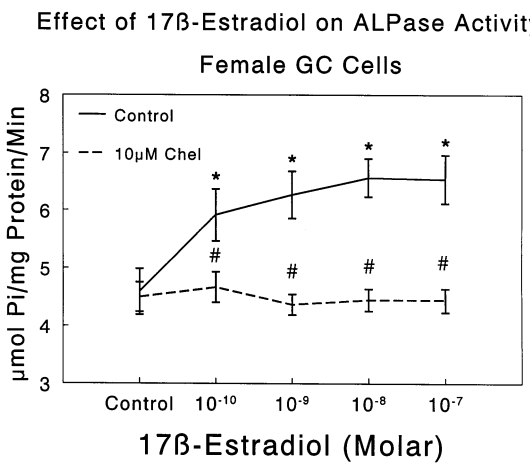

Fig. 3. Effect of the PKC inhibitor chelerythrine on 17β-estradiol-(E_2) induced alkaline phosphatase specific activity of growth zone chondrocytes. Confluent, fourth passage growth zone cells (GC) from female rats were treated for 24 h with control media or media containing 10^{-10}–10^{-7} M E_2 in the presence or absence of 10 μM chelerythrine. At harvest, alkaline phosphatase specific activity 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. $^{*}P < 0.05$, E_2 treatment vs. control; $^{\#}P < 0.05$, Chel vs. control.

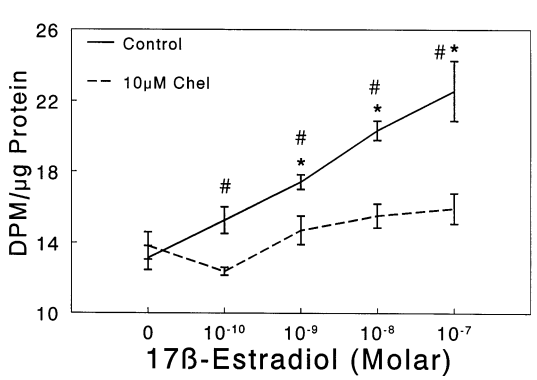

Fig. 4. Effect of the PKC inhibitor chelerythrine on 17β-estradiol-(E_2) induced proteoglycan sulfation of growth zone chondrocytes. Confluent, fourth passage growth zone cells (GC) from female rats were treated for 24 h with control media or media containing 10^{-10}–10^{-7} M E_2 in the presence or absence of 10 μM chelerythrine. 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. $^{*}P < 0.05$, E_2 treatment vs. control; $^{\#}P < 0.05$, Chel vs. control.

Table 1
Effect of 17β-estradiol receptor agonist diethylstilbesterol (DES) and antagonist ICI 182780 (ICI) on PKC activity in female resting zone chondrocytes[a]

	Protein kinase C specific activity (pmol Pi/μg protein/min)	
Inhibitor [M]	DES	ICI
Control-E_2	0.45 ± 0.04	0.45 ± 0.04
$+10^{-9}$	0.44 ± 0.04	0.42 ± 0.02
$+10^{-8}$	0.40 ± 0.02	0.40 ± 0.03
$+10^{-7}$	0.45 ± 0.05	0.41 ± 0.04
Control + 10^{-7} M E_2	2.10 ± 0.16*	2.10 ± 0.16*
$+10^{-9}$	2.07 ± 0.17*	2.01 ± 0.11*
$+10^{-8}$	2.11 ± 0.18*	2.02 ± 0.08*
$+10^{-7}$	2.17 ± 0.12*	2.03 + 0.10*

[a] Each value is the mean ± SEM of six cultures from one of two experiments yielding comparable results. $^{*}P < 0.05$, $+E_2$ vs. $-E_2$ at each concentration of inhibitor.

3.2.3. Role of G-proteins

G-proteins mediate the effect of E_2 on PKC; however, neither Gi nor Gs is involved. The Gi inhibitor pertussis toxin (PTX) had no effect on PKC activity in E_2-treated RC cultures (Table 3). Similarly, cholera-toxin (CTX) had no effect. In contrast, PKC activity was reduced in RC cultures treated with E_2 in the presence of GDPβS (Fig. 8A). Cultures treated with either 10^{-8} M E_2 or E_2 + 100 ng/ml PTX in the presence of 1 μM GDPβS had PKC activity equivalent to that of control cultures. At higher concentrations of the inhibitor, PKC was reduced to levels below baseline whether or not E_2 was present. This was noted at all times examined (Fig. 8B).

3.2.4. Role of tyrosine kinases

Tyrosine kinase activity is not required for the E_2-dependent stimulation of PKC activity (Fig. 9). Genistein (10 μM) had no effect on PKC activity in control or E_2-treated RC cultures. The lack of an effect was noted

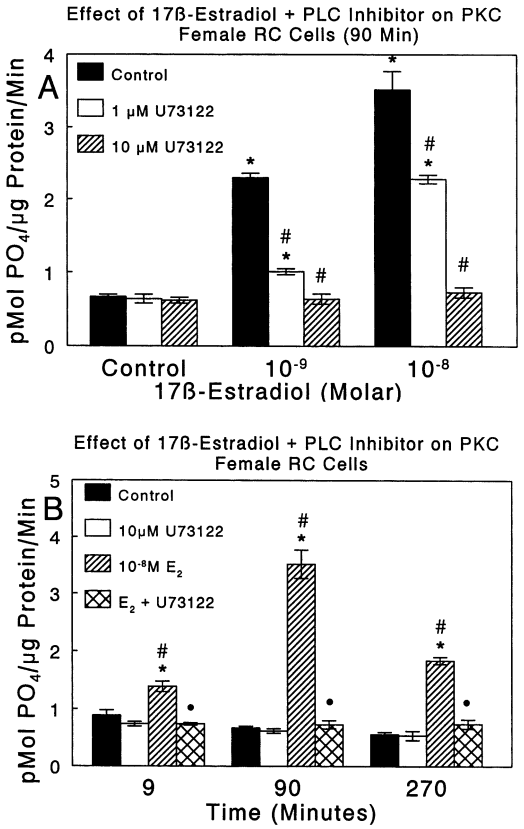

Fig. 5. Effect of the phospholipase C inhibitor, U73122, on PKC specific activity of resting zone chondrocytes treated with 17β-estradiol (E_2). Confluent, fourth passage resting zone cells (RC) from female rats were treated for 90 min with control media or media containing 10^{-9}–10^{-8} M E_2 in the presence or absence of 1 or 10 μM U73122 (Panel A). Alternatively, cultures were treated for 9, 90, or 270 min with control media or media containing 10 μM U73122, 10^{-8} M E_2, or E_2 + U73122 (Panel B). At harvest, PKC specific activity in the cell layer was determined as described in Section 2. Data represent the mean ± SEM of six cultures from one of three experiments, all with comparable results. Panel A: $^{*}P < 0.05$, E_2 treatment vs control; $^{\#}P < 0.05$, U73122 vs. control at a particular E_2 concentration. Panel B: $^{*}P < 0.05$, E_2 treatment vs. control; $^{\#}P < 0.05$, treatment vs. U73122; $^{\bullet}P < 0.05$, E_2 + U73122 vs. E_2 alone.

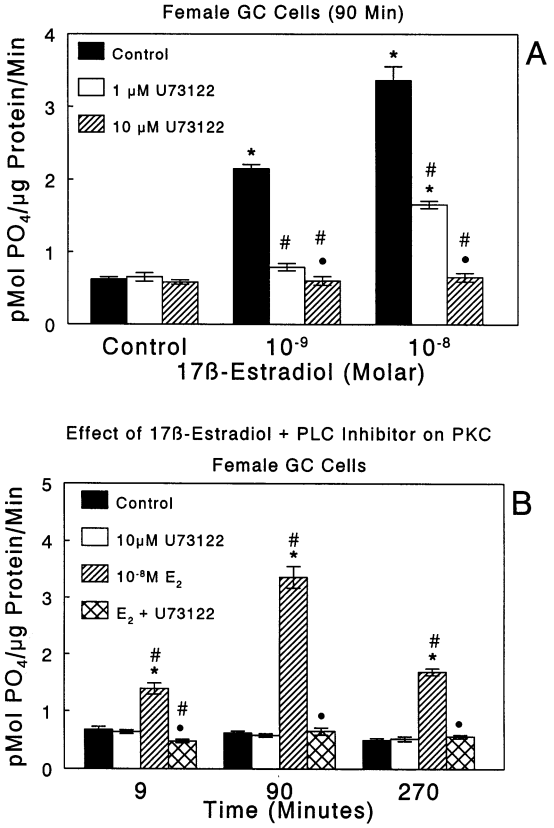

Fig. 6. Effect of the phospholipase C inhibitor, U73122, on PKC specific activity of growth zone chondrocytes treated with 17β-estradiol (E_2). Confluent, fourth passage growth zone cells (GC) from female rats were treated for 90 min with control media or media containing 10^{-9}–10^{-8} M E_2 in the presence or absence of 1 or 10 μM U73122 (Panel A). Alternatively, cultures were treated for 9, 90, or 270 min with control media or media containing 10 μM U73122, 10^{-8} M E_2, or E_2 + U73122 (Panel B). At harvest, PKC specific activity in the cell layer was determined as described in Section 2. Data represent the mean ± SEM of six cultures from one of three experiments, all with comparable results. Panel A: $^{*}P < 0.05$, E_2 treatment vs. control; $^{\#}P < 0.05$, U73122 vs. control at a particular E_2 concentration; $^{\bullet}P < 0.05$, 1 μM U731222 vs. 10 μM U73122 at a particular E_2 concentration. Panel B: $^{*}P < 0.05$, E_2 vs. control; $^{\#}P < 0.05$, E_2 treatment vs. U73122; $^{\bullet}P < 0.05$, E_2 + U73122 vs. E_2 alone.

Table 2
Effect of phosphatidylcholine-specific phospholipase C inhibitor D609 on 17β-estradiol activation of PKC in female resting zone chondrocytes[a]

Treatment (90 min)	Protein kinase C specific activity (pmol Pi/μg protein/min)		
	Control	10^{-8} M E_2	10^{-7} M E_2
D609 (μM)			
0	0.50 ± 0.08	1.69 ± 0.32	2.29 ± 0.14
1	0.55 ± 0.09	1.78 ± 0.37	2.20 ± 0.15
10	0.54 ± 0.06	1.67 ± 0.47	2.26 ± 0.21
100	0.52 ± 0.08	1.81 ± 0.38	2.19 ± 0.28

[a] Each value is the mean ± SEM of six cultures from one of two experiments yielding comparable results. No statistically significant differences were observed.

at all times examined and did not depend on the concentration of genistein used. Similar results were obtained when 0.1 or 1 μM genistein was used, and when the experiments were performed using GC cultures (data not shown).

3.2.5. Role of phospholipase A_2

Regulation of PKC activity by E_2 does not involve phospholipase A_2. Activation of phospholipase A_2 with mellitin inhibited basal PKC activity at all times examined, but had no effect on E_2-stimulated activity (Fig. 10A). Inhibition of PKC by 3 μg/ml melittin was noted at all times examined, and the effect was also observed in cultures treated with 0.03 or 0.3 μg/ml (data not

shown). Table 4 shows that another PLA_2 activator, mastoparan, had similar effects on PKC activity in E_2-treated RC cultures. In contrast to RC cultures, GC cultures treated with melittin or mastoparan alone exhibited increased PKC activity, but similar to RC cultures, there was no effect on PKC activity of E_2-treated GC cultures (data not shown).

Inhibition of phospholipase A_2 activity had no effect on PKC activity in control or E_2-treated cultures at 9 or 270 min, although there was a small increase in basal activity at 90 min in cells treated with 10 μM quinacrine (Fig. 10B). Similar results were seen when RC cultures were treated with 1, 10, or 100 μM quinacrine. Quinacrine caused a decrease in basal PKC in GC cultures, but had no effect on E_2-dependent enzyme activity (data not shown).

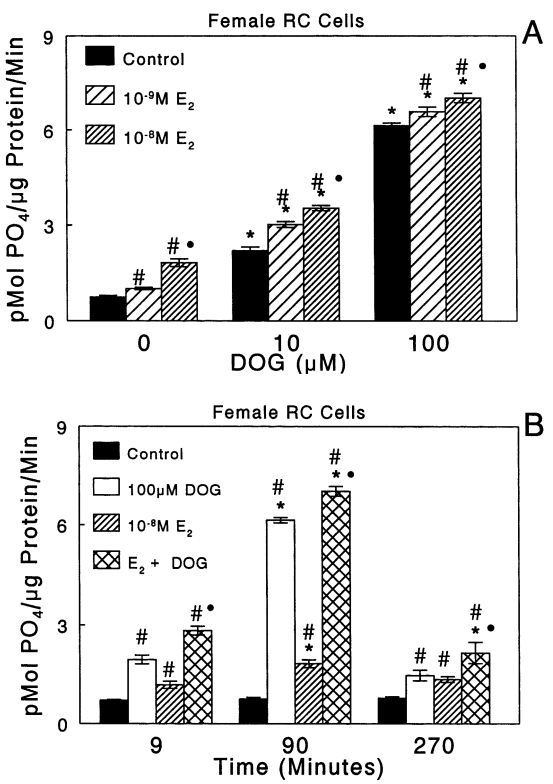

Fig. 7. Effect of dioctanoylglycerol (DOG) on PKC specific activity of resting zone chondrocytes treated with 17β-estradiol (E_2). Confluent, fourth resting growth zone cells (RC) from female rats were treated for 90 min with control media or media containing 10^{-9}–10^{-8} M E_2 in the presence or absence of 10 or 100 μM DOG (Panel A). Alternatively, cultures were treated for 9, 90, or 270 min with control media or media containing 100 μM DOG, 10^{-8} M E_2, or E_2 + DOG (Panel B). At harvest, PKC specific activity in the cell layer was determined as described in the Methods. Data are the mean ± SEM of six cultures from one of three experiments, all with comparable results. Panel A: $^{*}P < 0.05$, DOG treatment vs. control; $^{\#}P < 0.05$, E_2 vs. control for a particular concentration of DOG; $^{\bullet}P < 0.05$, 10^{-9} M E_2 vs. 10^{-8} M E_2. Panel B: $^{*}P < 0.05$, treatment vs. same treatment at 9 min; $^{\#}P < 0.05$, treatment vs. control for a particular time point; $^{\bullet}P < 0.05$, E_2 + DOG vs. DOG alone.

Table 3
Effect of G-protein inhibitors, pertussis toxin (PTX) and cholera toxin (CTX), on 17β-estradiol activation of PKC in female resting zone chondrocytes[a]

Treatment	Protein kinase C specific activity (pmol Pi/μg protein/min)	
	Control	10^{-7}M E_2
PTX (ng/ml)		
0	0.49 ± 0.03	1.84 ± 0.20
1	0.51 ± 0.06	1.90 ± 0.07
10	0.47 ± 0.03	1.82 ± 0.09
100	0.46 ± 0.03	1.85 ± 0.16
CTX (ng/ml)		
0	0.49 ± 0.03	1.84 ± 0.20
1	0.47 ± 0.03	1.84 ± 0.22
10	0.45 ± 0.04	1.87 ± 0.10
100	0.48 ± 0.03	1.85 ± 0.09

[a] Each value is the mean ± SEM of six cultures from one of two experiments yielding comparable results. No statistically significant differences were observed.

3.2.6. Role of cyclooxygenase and lipoxygenase

Arachidonic acid metabolites also do not mediate the effects of E_2 on PKC. Inhibition of prostaglandin production by indomethacin (Fig. 11A) caused a dose-dependent increase in E_2-treated cultures that was additive with the increase in basal activity in control cultures. In contrast, inhibition of lipoxygenase activity with NDGA had no effect on PKC activity in either control or E_2-treated RC cultures (Fig. 11B).

3.3. Membrane specificity of the E_2 effect

The effect of E_2 on PKC activity was membrane-specific and dose-dependent (Fig. 12A). Only PKC in matrix vesicles was increased when the membranes were incubated directly with the hormone. The effect was rapid, occurring within 3 min of exposure to 10^{-7} M hormone (Fig. 12B). Further increases were observed at 9 min at both 10^{-8} and 10^{-7} M, but by 90 min, the stimulatory effect of E_2 was no longer evident.

4. Discussion

This study demonstrates that E_2 exerts its effects on chondrocytes from female costochondral cartilage via PKC-mediated mechanisms. Inhibition of PKC mimicked the effects of E_2 on proliferation and reduced E_2-dependent stimulation of alkaline phosphatase specific activity and proteoglycan sulfation, indicating that proliferation, differentiation, and matrix synthesis were all affected. The effects were similar in RC and GC cells, consistent with our previous observation that both types of cartilage cells have receptors for E_2 and respond to the hormone in a comparable manner [9,35].

The fact that chelerythrine blocked the effects of E_2 on several aspects of chondrocyte physiology indicates the importance of the PKC pathway to the action of E_2 in cartilage cells. It is likely that E_2 modulates PKC-dependent phenotypic expression through a receptor-mediated mechanism since 17α-estradiol has no effect on phenotypic expression of RC or GC cells or on PKC activity in these cells [19,36]. Moreover, in the present study, we showed that inhibition of PKC had no effect on 17α-treated cells. It is unlikely that E_2 regulates PKC via traditional nuclear E_2 receptors, however, since neither the estrogen receptor agonist DES nor the antagonist ICI 182780 [41] had an effect on PKC. Moreover, neither compound affected E_2-dependent stimulation of PKC.

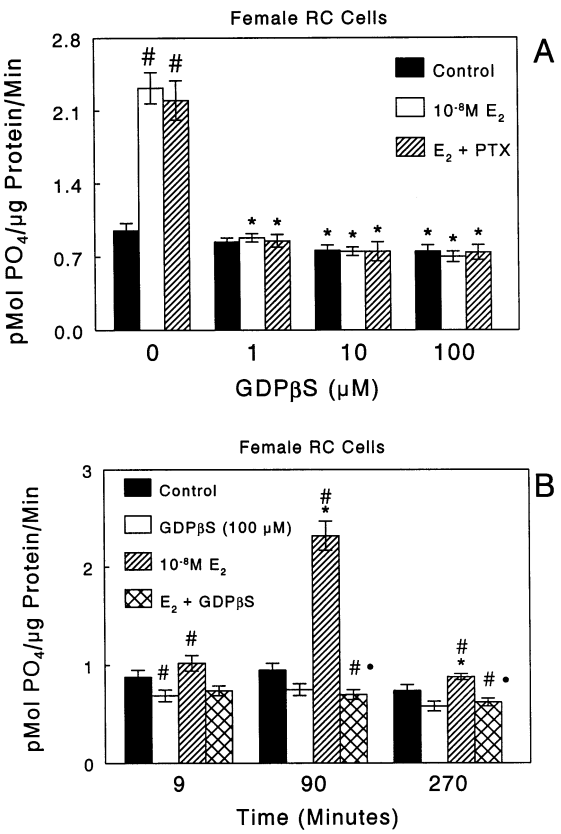

Fig. 8. Effect of G-protein inhibitors on PKC specific activity of resting zone chondrocytes (RC) treated with 17β-estradiol (E_2). Confluent, fourth passage resting zone cells (RC) from female rats were treated for 90 min with control media or media containing 10^{-8} M E_2, or 10^{-8} M E_2 + 100 ng/ml pertussis toxin (PTX) in the presence or absence of 1, 10, or 100 µM GDPβS (Panel A). Alternatively, cultures were treated for 9, 90, or 270 min with control media or media containing 100 µM GDPβS, 10^{-8} M E_2, or E_2 + GDPβS (Panel B). At harvest, PKC specific activity in the cell layer was determined as described in Section 2. Data are the mean ± SEM of six cultures from one of three experiments, all with comparable results. Panel A: $^{*}P < 0.05$, GDPβS vs. control; $^{\#}P < 0.05$, E_2 or E_2 + PTX treatment vs. control. Panel B: $^{*}P < 0.05$, treatment vs. same treatment at the other time points; $^{\bullet}P < 0.05$, E_2 + GDPβS vs. E_2 alone.

The mechanisms by which E_2 stimulates PKC activity appear to be membrane-associated. The response of PKC activity to E_2 is rapid, occurring within 3 min, and is independent of gene transcription or protein synthesis [58]. The isoform responsible is PKCα, a calcium- and phospholipid-dependent enzyme [64,65], suggesting that rapid changes in Ca ion flux or phospholipid metabolism could contribute to changes in activity noted in the present study. Rapid movement of Ca ions in response to E_2 have been observed in a number of cell types [15,23,66], suggesting that Ca ion flux may be regulated in chondrocytes as well. 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 [67]. It is unlikely that such a mechanism is involved in the present study, however, since E_2 concentrations of 0.1–100 nM elicit the PKC response.

Phospholipid metabolism is involved in the E_2-dependent stimulation of PKC. Inhibition of PLC blocked E_2-stimulation of PKC activity in a dose-dependent manner at all time points examined, implicating a PLC-dependent pathway in the mechanism. Inhibition of tyrosine kinase was without effect, suggesting that the PLC involved is not activated through tyrosine phosphorylation. The data presented here indicate that the PLC responsible for E_2-dependent stimulation of PKC is PI-specific rather than PC-specific. This is supported by the fact that the product of the action of PLC on phosphatidylinositol, diacylglycerol, causes an increase in PKC that is additive with the increase caused by E_2. Moreover, the PLC involved is coupled to a G-protein that is insensitive to pertussis toxin and choleratoxin. One possibility is PLCβ, which is coupled to the pertussis toxin-insensitive Gq.

While PLC is involved in the mechanism, phospholipase A_2 is not. As we have shown previously, activation of phospholipase A_2 caused a decrease in basal PKC in RC cells and an increase in basal PKC in GC cells [58,68]. However, neither melittin nor mastoparan had an effect on PKC activity in E_2-treated cells. Similarly, inhibition of phospholipase A_2 with quinacrine caused the expected increase in PKC in RC cells and decrease in GC cells [58,63], but it had no effect on PKC activity in E_2-treated cells.

E_2 causes a rapid increase in arachidonic acid turnover in costochondral chondrocytes [35], indicating that it regulates both deacylation and reacylation of arachidonic acid. Since arachidonic acid is usually located on the C-2 carbon of the phospholipid glycerol backbone, it is evident that E_2 regulates phospholipase A_2. This suggests that E_2 may use a phospholipase A_2-dependent signaling pathway to mediate effects in addition to PKC. It is also possible that metabolites of arachidonic acid might modulate the effect of E_2 on PKC, but our results indicate that this is not the case.

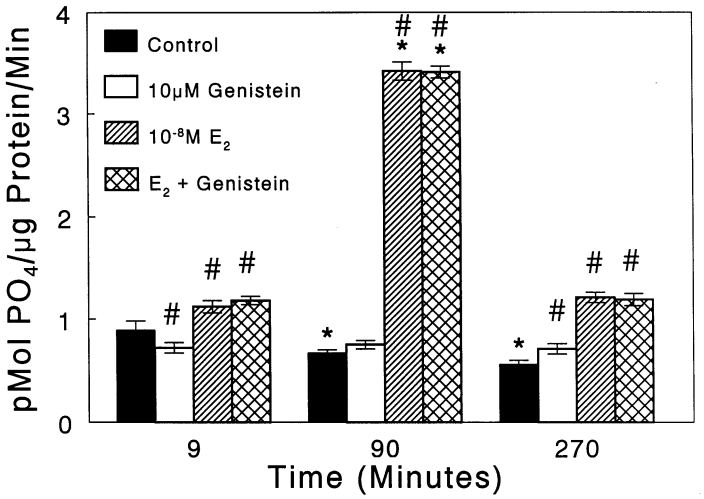

Fig. 9. Effect of the tyrosine kinase inhibitor genistein on PKC specific activity of resting zone chondrocytes treated with 17β-estradiol (E_2). Confluent, fourth passage resting zone cells (RC) from female rats were treated for 9, 90, or 270 min with control media or media containing 10 μM genistein, 10^{-8} M E_2, or genistein + E_2. At harvest, PKC specific activity in the cell layer was determined as described in Section 2. Data are the mean ± SEM of six cultures from one of three experiments, all with comparable results. $^{*}P < 0.05$, treatment vs. same treatment at 9 min; $^{\#}P < 0.05$, treatment vs. control for a particular time point.

Inhibition of prostaglandin production by indomethacin resulted in an increase in basal PKC [63]. However, it did not increase the effect of E_2 beyond its effect on basal enzyme activity. Leukotrienes also do not appear to play a role, since inhibition of lipoxygenase had no effect either on basal activity (unpublished data) or on E_2-stimulated PKC.

E_2 only stimulates PKC in chondrocytes from female rats [58], even though both male and female chondrocytes have classical E_2 receptors [9]. However, only plasma membranes and matrix vesicles isolated from cultures of female chondrocytes exhibited a change in fluidity in response to E_2 [18]. This suggested that the gender-specific effects of the hormone might be mediated through a nongenomic, membrane-mediated mechanism. The results of the present study indicate that at least part of the E_2-dependent increase in PKC is nongenomic and membrane-mediated. Direct incubation of matrix vesicles with E_2 resulted in increased PKC, even though these extracellular organelles contain no DNA or RNA.

We previously observed that when chondrocyte cultures are incubated with E_2, the increase in alkaline phosphatase activity is targeted to matrix vesicles rather than the plasma membrane [35]. Because these experiments had involved a 24-h incubation, we assumed that E_2 upregulated alkaline phosphatase gene expression and new matrix vesicle production. The present study suggests that some of the increase in matrix vesicle alkaline phosphatase may also be due to the rapid effects of E_2 on PKC.

Our results also suggest that E_2 may regulate PKC via two separate, but interrelated, mechanisms. The hormone causes a rapid increase in matrix vesicle PKC within 3 min, reaching maximal activity by 9 min. This effect is resolved by 90 min. The rapid response is also seen in intact cultures, but maximal activity occurs at 90 min. E_2 does not cause PKC to be translocated to the plasma membrane [69], so this latter effect is probably due to cytosolic PKCα.

The recognition that E_2 exerts some of its actions via rapid membrane-mediated mechanisms is relatively new [15,67,70,71]. 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 effects of E_2 described here are distinct from those previously reported for either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$, whether one examines phospholipase A_2 activity [72,73], arachidonic acid turnover [74], membrane fluidity [75], or PKC activity.

Although 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ have membranous effects on chondrocytes, their pathways of action are different from that of E_2 [31,58,63]. 1,25-$(OH)_2D_3$ activates PKC in GC cells maximally at 9 min via a PI-PLC-dependent mechanism [31]; 1,25-$(OH)_2D_3$ has no effect on PKC in RC cells. 24,25-$(OH)_2D_3$ activates PKC of RC, but not GC, maximally at 90 min via a PLC-independent mechanism [63]. Similar to 1,25-$(OH)_2D_3$, E_2 activates PKC through a pertussis toxin-independent, PI-PLC-dependent pathway. However, unlike 1,25-$(OH)_2D_3$, E_2 does not display cell maturation-specific effects and activates PKC maximally at 90 min in both GC and RC cells [19].

Unlike vitamin D_3 metabolites, which stimulate plasma membrane PKC and inhibit matrix vesicle PKC, this study has shown that E_2 has no direct effect on plasma membrane PKC activity, and it stimulates

matrix vesicle PKC activity. The difference between the actions of E_2 and vitamin D_3 metabolites on plasma membrane PKC may be explained by a direct interaction between 1,25-$(OH)_2D_3$ and PKC molecules, as shown previously [76], which may not exist between E_2 and PKC. Another possibility might involve specific membrane receptors for 1,25-$(OH)_2D_3$ and E_2. GC cells and their matrix vesicles, have a specific membrane receptor for 1,25-$(OH)_2D_3$ [33,34]. 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 [22,71]. Differences in PKC activation between 1,25-$(OH)_2D_3$ and E_2 may reflect differences in other signaling enzymes coupled to the 1,25-$(OH)_2D_3$ receptor, such as phospholipase A_2.

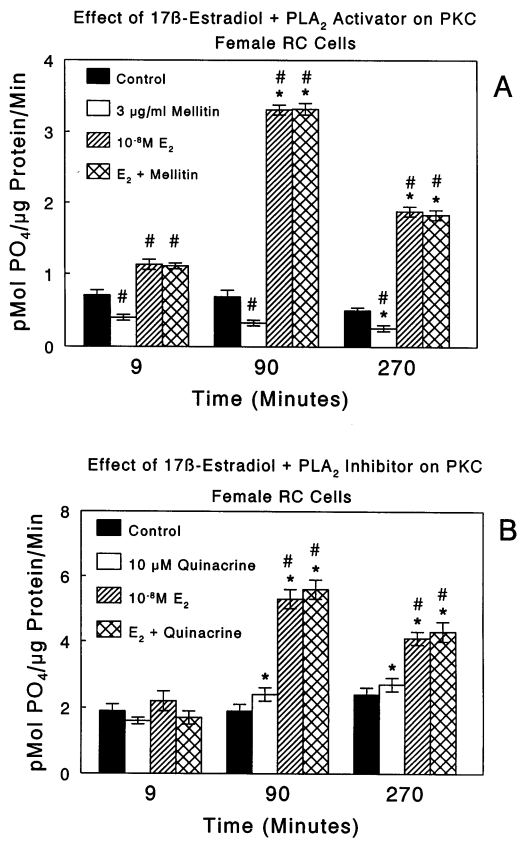

Fig. 10. Effect of the phospholipase A_2 activator, melittin, and the phospholipase A_2 inhibitor, quinacrine, on PKC specific activity of resting zone chondrocytes treated for various periods of time with 17β-estradiol (E_2). Confluent, fourth passage resting zone cells (RC) from female rats were treated for 9, 90, or 270 min with control media or media containing 3 µg/ml melittin, 10^{-8} M E_2, or E_2 + melittin (Panel A). Alternatively, similarly prepared cultures were treated for the same periods of time with control media or media containing 10 µM quinacrine, 10^{-8} M E_2 or E_2 + quinacrine (Panel B). At harvest PKC activity in the cell layer was determined as described in Section 2. Data represent the mean ± SEM of six cultures from one of three experiments, all with comparable results. $^{*}P < 0.05$, treatment vs. same treatment at 9 min; $^{\#}P < 0.05$, treatment vs. control for a particular time point.

Table 4
Effect of the phospholipase A_2 activator, mastoparan, on 17β-estradiol activation of PKC in female resting zone chondrocytes[a]

	Protein kinase C specific activity (pmol Pi/µg protein/min)		
	Incubation time (min)		
Treatment	9	90	270
Control	0.44 ± 0.03	0.42 ± 0.04	0.50 ± 0.03
Mastoparan (20 µg/ml)	0.24 ± 0.04^{b}	0.23 ± 0.05^{b}	0.25 ± 0.04^{b}
10^{-8}M E_2	$0.96 \pm 0.05^{b,d}$	$3.74 \pm 0.09^{b,c,d}$	$3.77 \pm 0.09^{b,c,d}$
Mastoparan + E_2	$0.98 \pm 0.06^{b,d}$	$3.77 \pm 0.05^{b,c,d}$	$1.74 \pm 0.08^{b,c,d,e}$

[a] Each value is the mean ± SEM of six cultures from one of two experiments yielding comparable results. $^{b}P < 0.05$, treatment vs. control at each time point; $^{c}P < 0.05$, vs. 9 min for each treatment; $^{d}P < 0.05$, vs. mastoparan treatment alone; $^{e}P < 0.05$, vs. E_2 treatment alone.

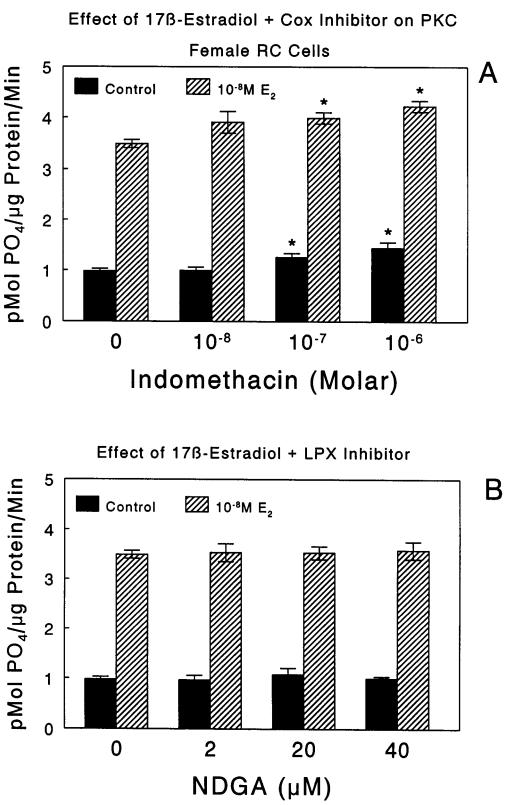

Fig. 11. Effect of the cyclooxygenase inhibitor, indomethacin, and the lipoxygenase inhibitor, NDGA, on PKC specific activity of resting zone chondrocytes treated with 17β-estradiol (E_2). Confluent, fourth passage resting zone cells (RC) from female rats were treated for 90 min with control media or media containing 10^{-8} M E_2 in the presence or absence of 10^{-8}–10^{-6} M indomethacin (Panel A) or 2, 20, or 40 µM NDGA (Panel B). 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 three esperiments, all with comparable results. $^{*}P < 0.05$, treatment with indomethacin vs. control.

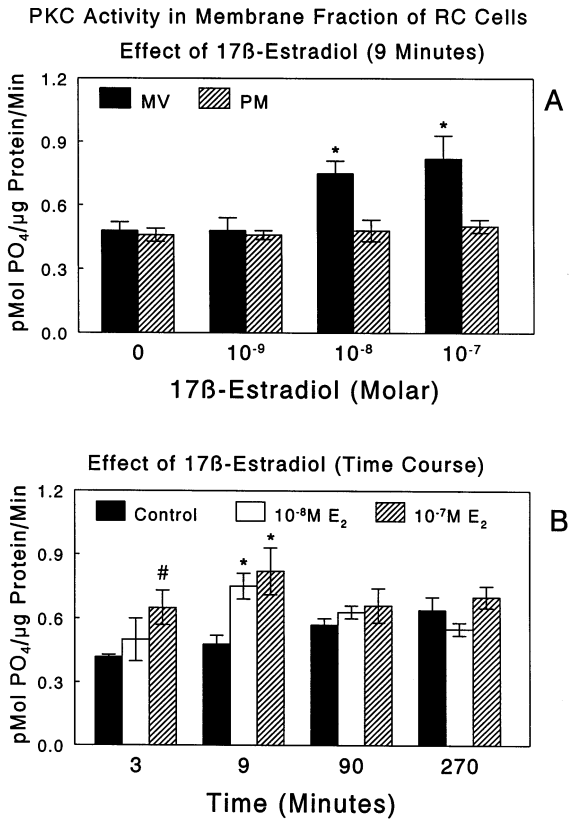

Fig. 12. Direct effect of 17β-estradiol (E_2) on PKC activity in isolated matrix vesicles and plasma membranes from resting zone chondrocyte (RC) cultures. Matrix vesicles (MV) and plasma membranes (PM) were isolated, incubated for 9 min with either control media or media containing 10^{-9}–10^{-7} M E_2 (Panel A). Isolated MV were also treated for 3, 9, 90, and 270 min with control media or media containing 10^{-8} or 10^{-7} M E_2 (Panel B). At harvest, PKC specific activity in the cell layer was determined as described in Section 2. Data represent the mean ± SEM of PKC activity in membranes from a representative experiment ($n = 6$) that was repeated twice with comparable results. $^{*}P < 0.05$, E_2 treatment vs control (Panel A); $^{\#}P < 0.05$, 10^{-7} M E_2 vs. control for a particular time point (Panel B).

Regardless of these differences, this study makes clear that E_2 exerts membrane-associated effects in chondrocytes. The membranous effect of E_2 regulates different aspects of chondrocyte physiology, including proliferation, differentiation, and matrix production. These findings indicate the importance of the membrane effects as mediators of E_2 action in chondrocytes and suggest the existence of a specific membrane receptor for E_2 in chondrocytes.

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References

[1] Z. Schwartz, J. Meincke, E. Nasatzky, D.D. Dean, B.D. Boyan, Estrogen regulation of endochondral bone formation, in: A. Ornoy (Ed.), Animal Models of Human Related Calcium Metabolic Disorders, CRC Press, Boca Raton, FL, 1995, pp. 149–164.

[2] A. Ornoy, S. Giron, R. Aner, M. Goldstein, B.D. Boyan, Z. Schwartz, Gender dependent effects of testosterone and 17 beta-estradiol on bone growth and modelling in young mice, Bone Miner. 24 (1994) 43–58.

[3] Z. Schwartz, W.A. Soskolne, T. Neubauer, M. Goldstein, S. Adi, A. Ornoy, Direct and sex-specific enhancement of bone formation and calcification by sex steroids in fetal mice long bone in vitro (biochemical and morphometric study), Endocrinology 129 (1991) 1167–1174.

[4] T.K. Gray, B. Lipes, T. Linkhart, S. Mohan, D. Baylink, Transforming growth factor beta mediates the estrogen induced inhibition of UMR106 cell growth, Connect. Tissue Res. 20 (1989) 23–32.

[5] A.R. Means, B.W. O'Malley, Mechanism of estrogen action: early transcriptional and translational events, Metab. Clin. Exp. 21 (1972) 357–370.

[6] G.G. Kuiper, E. Enmark, M. Pelto-Huikko, S. Nilsson, J.A. Gustafsson, Cloning of a novel estrogen receptor expressed in rat prostate and ovary, Proc. Natl. Acad. Sci. USA 93 (1996) 5925–5930.

[7] N. Dayani, M.T. Corvol, P. Robel, B. Eychenne, B. Moncharmont, L. Tsagris, R. Rappaport, Estrogen receptors in cultured rabbit articular chondrocytes: influence of age, J. Steroid Biochem. 31 (1988) 351–356.

[8] H. Pinus, A. Ornoy, N. Patlas, P. Yaffe, Z. Schwartz, Specific beta estradiol binding in cartilage and serum from young mice and rats is age dependent, Connect. Tissue Res. 30 (1993) 85–98.

[9] E. Nasatzky, Z. Schwartz, W.A. Soskolne, B.P. Brooks, D.D. Dean, B.D. Boyan, A. Ornoy, Evidence for receptors specific for 17b-estradiol and testosterone in chondrocyte cultures, Connect. Tissue Res. 30 (1994) 277–294.

[10] B.A. Monaghan, F.S. Kaplan, C.R. Lyttle, M.D. Fatton, S.D. Boden, J.G. Haddad, Estrogen receptors in fracture healing, Clin. Orthop. Rel. Res. 280 (1992) 277–280.

[11] I. Nemere, M.C. Farach-Carson, Membrane receptors for steroid hormones: a case for specific cell surface binding sites for vitamin D metabolites and estrogens, Biochem. Biophys. Res. Commun. 248 (1998) 443–449.

[12] A. Nadal, J.M. Rovira, O. Laribi, T. Leon-Quinto, E. Andreu, C. Ripoll, B. Soria, Rapid insulinotropic effect of 17b-estradiol via a plasma membrane receptor, FASEB J. 12 (1998) 1341–1348.

[13] T.C. Pappas, B. Gametchu, J. Yannariello-Brown, T.J. Collins, C.S. Watson, Membrane estrogen receptors in GH3/B6 cells are associated with rapid estrogen-induced release of prolactin, Endocrine 2 (1994) 813–822.

[14] A. Revelli, M. Massobrio, J. Tesarik, Nongenomic actions of steroid hormones in reproductive tissues, Endocrine Rev. 19 (1998) 3–17.

[15] P.G. Mermelstein, J.B. Becker, D.J. Surmeier, Estradiol reduces calcium currents in rat neostriatal neurons via a membrane receptor, J. Neurosci. 16 (1996) 595–604.

[16] M. Lieberherr, B. Grosse, M. Kachkache, S. Balsan, Cell signaling and estrogens in female rat osteoblasts: a possible involvement of unconventional nonnuclear receptors, J. Bone Miner. Res. 8 (1993) 1365–1376.
[17] K.D. Brubaker, C.V. Gay, Specific binding of estrogen to osteoclast surfaces, Biochem. Biophys. Res. Commun. 200 (1994) 899–907.
[18] Z. Schwartz, P.A. Gates, E. Nasatzky, V.L. Sylvia, J. Mendez, D.D. Dean, B.D. Boyan, Effect of 17b-estradiol on chondrocyte membrane fluidity and phospholipid metabolism is membrane-specific, sex-specific, and cell maturation-dependent, Biochim. Biophys. Acta 1282 (1996) 1–10.
[19] V.L. Sylvia, T. Hughes, D.D. Dean, B.D. Boyan, Z. Schwartz, 17β-Estradiol regulation of protein kinase C activity in chondrocytes is sex-dependent and involves nongenomic mechanisms, J. Cell Physiol. 176 (1998) 435–444.
[20] B.D. Boyan, Z. Schwartz, D.L. Carnes, Jr, V. Ramirez, The effects of vitamin D metabolites on the plasma and matrix vesicle membranes of growth and resting cartilage cells in vitro, Endocrinology 122 (1988) 2851–2860.
[21] V.D. Ramirez, J. Zheng, K.M. Siddique, Membrane receptors for estrogen, progesterone, and testosterone in the rat brain: fantasy or reality, Cell. Mol. Neurobiol. 16 (1996) 175–198.
[22] T.C. Pappas, B. Gametchu, C.S. Watson, Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding, FASEB J. 9 (1994) 404–410.
[23] M. Wehling, Specific, nongenomic actions of steroid hormones, Ann. Rev. Physiol. 59 (1997) 365–393.
[24] I. Pailler-Rodde, H. Garcin, P. Higueret, Effect of retinoids on protein kinase C activity and on the binding characteristics of the tri-iodothyronine nuclear receptor, J. Endocr. 128 (1991) 245–251.
[25] S. Morelli, R. Boland, A.R. de Boland, 1,25(OH)2-vitamin D3 stimulation of phospholipases C and D in muscle cells involves extracellular calcium and a pertussis-sensitive G protein, Mol. Cell Endocrinol. 122 (1996) 207–211.
[26] T. Magda, V. Lloyd, Protein kinase C activity and messenger RNA modulation by estrogen in normal and neoplastic rat pituitary tissue, Lab. Invest. 68 (1993) 472–480.
[27] H. Endoh, H. Sasaki, K. Maruyama, K. Takeyama, I. Waga, T. Shimizu, S. Kato, H. Kawashima, Rapid activation of MAP kinase by estrogen in the bone cell line, Biochem. Biophys. Res. Commun. 235 (1997) 99–102.
[28] M. Inoue, Y. Nishizuka, Studies on a cyclic nucleotide-independent protein kinase and its proenzymes on mammalian tissues, J. Biol. Chem. 252 (1977) 7610–7616.
[29] K. Rajkumar, Effect of protein kinase C inhibitor on estradiol-induced deoxyribonucleic acid synthesis in rats, Steroids 58 (1993) 100–105.
[30] J. Fujimoto, M. Hori, S. Ichigo, S. Morishita, T. Tamaya, Estrogen induces expression of c-fos and c-jun via activation of protein kinase C in an endometrial cancer cell line and fibroblasts derived from human uterine endometrium, Gynecol. Endocrinol. 10 (1996) 109–118.
[31] V.L. Sylvia, Z. Schwartz, L. Schuman, R.T. Morgan, S. Mackey, R. Gomez, B.D. Boyan, Maturation-dependent regulation of protein kinase C activity by vitamin D3 metabolites in chondrocyte cultures, J. Cell Physiol. 157 (1993) 271–278.
[32] V.L. Sylvia, Z. Schwartz, E.B. Ellis, S.H. Helm, R. Gomez, D.D. Dean, B.D. Boyan, Nongenomic regulation of protein kinase C isoforms by the vitamin D metabolites 1a,25-(OH)2D3 and 24R,25-(OH)2D3, J. Cell Physiol. 167 (1996) 380–393.
[33] I. Nemere, Z. Schwartz, H. Pedrozo, V.L. Sylvia, D.D. Dean, B.D. Boyan, Identification of a membrane receptor for 1,25-dihydroxy vitamin D3 which mediates rapid activation of protein kinase C, J. Bone Miner. Res. 13 (1998) 1353–1359.
[34] H.A. Pedrozo, Z. Schwartz, S. Rimes, V.L. Sylvia, I. Nemere, G.H. Posner, D.D. Dean, B.D. Boyan, Physiological importance of the 1,25-(OH)2D3 membrane receptor and evidence for a membrane receptor specific for 24,25-(OH)2D3, J. Bone Miner. Res. 14 (1999) 856–867.
[35] E. Nasatzky, Z. Schwartz, B.D. Boyan, W.A. Soskolne, A. Ornoy, Sex-dependent effects of 17-b-estradiol on chondrocyte differentiation in culture, J. Cell Physiol. 154 (1993) 359–367.
[36] E. Nasatzky, Z. Schwartz, W.A. Soskolne, B.P. Brooks, D.D. Dean, B.D. Boyan, A. Ornoy, Sex steroid enhancement of matrix production by chondrocytes is sex and cell maturation specific, Endocrine J. 2 (1994) 207–215.
[37] A. Carrascosa, M.T. Corvol, L. Tsagris, R. Rappaport, Biological effect of estradiol on phosphatase activities in rabbit cultured chondrocytes (abstract), Pediatr. Res. 25 (1981) 1542.
[38] O. Blanchard, L. Tsagris, R. Rappaport, G. Duval-Beaupere, M.T. Corvol, Age-dependent responsiveness of rabbit and human cartilage cells to sex steroids in vitro, J. Steroid Biochem. Mol. Biol. 40 (1991) 711–716.
[39] D.M. Perez, M.D. DeYoung, R.M. Graham, Coupling of expressed alpha 1B- and alpha 1D-adrenergic receptor to multiple signaling pathways is both G protein and cell type specific, Mol. Pharmacol. 44 (1993) 784–795.
[40] D. Church, S. Braconi, M. Vallotton, U. Lang, Protein kinase C-mediated phospholipase A2 activation, platelet-activating factor generation and prostacyclin release in spontaneously beating rat cardiomyocytes, Biochem. J. 290 (1993) 477–482.
[41] A.E. Wakeling, Use of pure antioestrogens to elucidate the mode of action of oestrogens, Biochem. Pharmacol. 49 (1995) 1545–1549.
[42] J.M. Herbert, J.M. Augereau, J. Gleye, J.P. Maffrand, Chelerythrine is a potent and specific inhibitor of protein kinase C, Biochem. Biophys. Res. Commun. 172 (1990) 993–999.
[43] T. Akiyama, J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya, Y. Fukami, Genistein, a specific inhibitor of tyrosine-specific protein kinases, J. Biol. Chem. 262 (1987) 5592–5595.
[44] J. Bleasdale, G.L. Bundy, S. Bunting, F.A. Fitzpatrick, R.M. Huff, F.F. Sun, J.E. Pike, Inhibition of phospholipase C-dependent processes by U73,122, Adv. Prostag. Thrombox. Leuk. Res. 19 (1989) 590–593.
[45] K. Muller-Decker, Interruption of TPA-induced signals by an antiviral and antitumoral xanthate compound: inhibition of a phospholipase C-type reaction, Biochem. Biophys. Res. Commun. 162 (1989) 198–205.
[46] M. Jang, L. Cai, G.O. Udeani, K.V. Slowing, C.F. Thomas, C.W. Beecher, H.H. Fong, N.R. Farnsworth, A.D. Kinghorn, R.G. Mehta, R.C. Moon, J.M. Pezzuto, Cancer chemopreventive activity of resveratrol, a natural product derived from grapes, Science 275 (1997) 218–220.
[47] A. Argiolas, J.J. Pisano, Facilitation of phospholipase A2 activity by mastoparans, a new class of mast cell degranulating peptides from wasp venom, J. Biol. Chem. 258 (1983) 13697–13702.
[48] E. Habermann, Bee and wasp venoms, Science 177 (1972) 314–322.
[49] T. Neichi, Y. Koshihara, S. Murota, Inhibitory effect of esculetin on 5-lipoxygenase and leukotriene biosynthesis, Biochim. Biophys. Acta 753 (1983) 130–132.
[50] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[51] B.D. Boyan, Z. Schwartz, L.D. Swain, D.L. Carnes, Jr, T. Zislis, Differential expression of phenotype by resting zone and growth region costochondral chondrocytes in vitro, Bone 9 (1988) 185–194.

[52] B.D. Boyan, Z. Schwartz, L.D. Swain, In vitro studies on the regulation of endochondral ossification by vitamin D, Crit. Rev. Oral Biol. Med. 3 (1992) 15–30.
[53] Z. Schwartz, D.L. Schlader, V. Ramirez, M.B. Kennedy, B.D. Boyan, Effects of vitamin D metabolites on collagen production and cell proliferation of growth zone and resting zone cartilage cells in vitro, J. Bone Miner. Res. 4 (1989) 199–207.
[54] B.D. Boyan, Z. Schwartz, L.D. Swain, Cell maturation-specific autocrine/paracrine regulation of matrix vesicles [Review], Bone Miner. 17 (1992) 263–268.
[55] J.P. Bretaudiere, T. Spillman, Alkaline phosphatases, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, vol. 4, Verlag Chemica, Weinheim, 1984, pp. 75–92.
[56] L.V. Hale, M.L. Kemick, R.E. Wuthier, Effect of vitamin D metabolites on the expression of alkaline phosphatase activity by epiphyseal hypertrophic chondrocytes in primary cell culture, J. Bone Miner. Res. 1 (1986) 489–495.
[57] R.J. O'Keefe, J.E. Puzas, J.S. Brand, R.N. Rosier, Effects of transforming growth factor-beta on matrix synthesis by chick growth plate chondrocytes, Endocrinology 122 (1988) 2953–2961.
[58] V.L. Sylvia, Z. Schwartz, D.B. Curry, Z. Chang, D.D. Dean, B.D. Boyan, 1,25-(OH)2D3 regulates protein kinase C activity through two phospholipid-independent pathways involving phospholipase A2 and phospholipase C in growth zone chondrocytes, J. Bone Miner. Res. 13 (1998) 559–569.
[59] R.M. Bell, Y. Hannun, C. Loomis, Mixed micelle assay of protein kinase C, Methods Enzymol. 124 (1986) 353–359.
[60] M.J. Berridge, Inositol lipids and cell proliferation, Biochim. Biophys. Acta 907 (1987) 33–45.
[61] J.R. Vane, Y.S. Bakhle, R.M. Botting, Cyclooxygenases 1 and 2, Ann. Rev. Pharmacol. Toxicol. 38 (1998) 97–120.
[62] Z. Schwartz, D.L. Schlader, L.D. Swain, B.D. Boyan, Direct effects of 1,25-dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 on growth zone and resting zone chondrocyte membrane alkaline phosphatase and phospholipase-A2 specific activities, Endocrinology 123 (1988) 2878–2884.
[63] S.H. Helm, V.L. Sylvia, T. Harmon, D.D. Dean, B.D. Boyan, Z. Schwartz, 24,25-(OH)2D3 regulates protein kinase C through two distinct phospholipid-dependent mechanisms, J. Cell Physiol. 169 (1996) 509–521.
[64] H. Hug, T.F. Sarre, Protein kinase C isoenzymes: divergence in signal transduction?, Biochem. J. 291 (1993) 329–343.
[65] S. Ohno, Y. Akita, A. Hata, S. Osada, K. Kubo, Y. Konno, K. Akimoto, K. Mizuno, T. Saido, T. Kuroki, Structural and functional diversities of a family of signal transducing protein kinases, protein kinase C family; two distinct classes of PKC, conventional cPKC and Novel nPKC, Adv. Enzyme Reg. 31 (1991) 287–303.
[66] G. Dayanithi, L. Tapia-Arancibia, Rise in intracellular calcium via a nongenomic effect of allopregnanolone in fetal rat hypothalamic neurons, J. Neurosci. 16 (1996) 130–136.
[67] M.A. Valverde, P. Rojas, J. Amigo, D. Cosmelli, P. Orio, M.I. Bahamonde, G.E. Mann, C. Vergara, R. Latorre, Acute activation of Maxi-K channels (hSlo) by estradiol binding to the beta subunit, Science 285 (1999) 1929–1931.
[68] B.D. Boyan, V.L. Sylvia, D.D. Dean, Z. Schwartz, Effects of 1,25-(OH)2D3 and 24,25-(OH)2D3 on protein kinase C in chondrocytes are mediated by phospholipase A2 and arachidonic acid, in: A.W. Norman, R. Bouillon, M. Thomasset (Eds.), Vitamin D: Chemistry, Biology, and Clinical Applications of the Steroid Hormone, University of California at Riverside, Riverside, CA, 1997, pp. 353–360.
[69] T. Keller, V.L. Sylvia, Y. Liu, D.D. Dean, Z. Chang, B.D. Boyan, Z. Schwartz, PGE2 mediates its 24,25-(OH)2D3-dependent effects on PKC via the EP1 receptor (abstract # 1264), J. Dent. Res. 77 (1998) 263.
[70] B.D. Boyan, D.D. Dean, V.L. Sylvia, Z. Schwartz, Nongenomic regulation of extracellular matrix events by vitamin D metabolites, J. Cell Biochem. 56 (1994) 331–339.
[71] G. Fiorelli, F. Gori, U. Frediani, F. Franceschelli, A. Tanini, C. Tosti-Guerra, S. Benvenuti, L. Gennari, L. Becherini, M.L. Brandi, Membrane binding sites and non-genomic effects of estrogen in cultured human pre-osteoclastic cells, J. Steroid Biochem. Mol. Biol. 59 (1996) 233–240.
[72] Z. Schwartz, B.D. Boyan, The effects of vitamin D metabolites on phospholipase A2 activity of growth zone and resting zone cartilage cells in vitro, Endocrinology 122 (1988) 2191–2198.
[73] L.D. Swain, Z. Schwartz, B.D. Boyan, 1,25-(OH)2D3 and 24,25-(OH)2D3 regulation of arachidonic acid turnover in chondrocyte cultures is cell maturation-specific and may involve direct effects on phospholipase A2, Biochim. Biophys. Acta 1136 (1992) 45–51.
[74] Z. Schwartz, L.D. Swain, V. Ramirez, B.D. Boyan, Regulation of arachidonic acid turnover by 1,25-(OH)2D3 and 24,25-(OH)2D3 in growth zone and resting zone chondrocyte cultures, Biochim. Biophys. Acta 1027 (1990) 278–286.
[75] L.D. Swain, Z. Schwartz, K. Caulfield, B.P. Brooks, B.D. Boyan, Nongenomic regulation of chondrocyte membrane fluidity by 1,25-(OH)2D3 and 24,25-(OH)2D3 is dependent on cell maturation, Bone 14 (1993) 609–617.
[76] S.J. Slater, M.B. Kelly, F.J. Taddeo, J.D. Larkin, M.D. Yeager, J.A. McLane, C. Ho, C.D. Stubbs, Direct activation of protein kinase C by 1-alpha 25-dihydroxyvitamin D3, J. Biol. Chem. 270 (1995) 6639–6643.