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Estrogen Modulation of Osteoblastic Cell-to-Cell Communication

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Abstract Two osteoblastic cell populations, calvarial and marrow stromal cells, were exposed to estrogen derivatives in vitro. The hormonal effect was monitored by following intracellular Ca^{+2} levels $[Ca^{+2}]_i$ and gap-junction communication. We measured fast changes in intracellular Ca^{+2} levels in response, of these cells, to the steroid hormones. The changes were dose dependent revealing maximal activity at 100 pM by 17-β-Estradiol and 1 nM by estradiol-CMO. Additionally, the effect of estrogen, on functional coupling of the cells, was measured using fluorescence dye migration and counting the number of neighboring cells coupled by gap junctions. An uncoupling effect was demonstrated in response of these cells to estrogen treatment. The quick stereospecific effect was achieved in the presence of 17-β-estradiol but not in the presence of 17-α-estradiol. These results suggest the involvement of plasma membrane receptors in addition to the already known nuclear receptors in transducing the hormone effects in the osteoblastic cells. J. Cell. Biochem. 69:282–290, 1998.

Key words: estrogen modulation; osteoblastic cells; plasma membrane receptors; nuclear receptors; gap junction communication

The sex steroids are known to play a crucial role in the process of skeletal development. Their effect was found to play a major role in puberty maturation, including skeletal changes. During aging, a decline in the levels of sex hormones is observed. In women a striking condition of estrogen-deficiency has been established as a major etiologic factor in postmenopausal osteoporosis, characterized by reduced bone formation [Riggs et al., 1972; Lindsay et al., 1980; Christiansen et al., 1981; Ivey and Baylink, 1981]. It was reported that osteoblastic cells from human [Arnet et al., 1987; Eriksen et al., 1988], rat [Komm et al., 1988], and mouse [Shamay et al., 1996; Ikegami et al., 1993] express high-affinity nuclear binding sites for 17β-Estradiol. It was shown that estrogen stimulates the formation of gap junctions as in the ovary [Merk et al., 1978] and in the myometrium [Dahl and Berger, 1978]. However, the mechanisms by which sex hormones exert their effects on bone are still unclear.

It is known that estrogen plays a major role in bone remodeling processes. This is due to alteration in cellular function of the osteoblastic and the osteoclastic cells' differentiation and function [Manolagas and Jilka, 1994]. In view of the fact that estrogen was shown to affect intracellular communication in non-skeletal organs [Merk et al., 1978; Dahl and Berger, 1978], it may be suggested that estrogen exerts an effect on skeletal tissue via a similar mechanism. The communication via gap-junctions between bone forming cells, osteoblasts, is a possible mechanism in transferring quick signals in response to hormonal triggering. Intracellular communication is mediated via the gap junction complexes consisting of clusters of transmembrane channels that mediate intercellular exchange of regulatory ions and small molecules. These channels are composed of pairs of protein subunits, connexins (Cx). Morphological studies have shown that gap junction channels, which span the transmembrane space, exist between bone cells [Doty, 1981; Shen et

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al., 1986]. Osteoblast-like cells were found to express connexin-43 (Cx43) and connexin-45 (Cx45) out of the 12 connexin types that were so far identified [Civitelli et al., 1993; Steinberg et al., 1994]. Gap-junctions were shown to be regulated by second messengers such as Ca^{+2} [Rose and Loewenstein, 1975] or cAMP [Saez et al., 1986; De Mello, 1988] through connexin phosphorylation [Saez et al., 1986; Swenson et al., 1990; Somogyi et al., 1989]. In addition, it was shown that pH could modulate the intercellular communication [Turin and Warner, 1977].

In the present work, we used primary calvaria and marrow-stromal derived osteoblastic cells to investigate the effect of estrogen derivatives on the inter-cellular communication via gap-junctions. These osteoblastic cells were originated and located at various anatomical sites of the skeleton. The calvarial osteoblasts were characterized by their response to PTH and their alkaline phosphatase level [Massas et al., 1993] as well as their membrane potential properties [Bingmann et al., 1988; Tetsch et al., 1989; Massas et al., 1990]. The marrow stromal osteoblasts were studied using the MBA-15 cell line. These cells have been studied for their osteogenic properties in vitro and in vivo. The growth, biochemical properties, mRNA expression, and cell attachment levels of MBA-15 to various matrix proteins, were previously described [Benayahu et al., 1989, 1991, 1994a,b,c, 1995a,b]. The osteogenic potential of MBA-15 cells to form bone tissue in vivo was demonstrated by employing diffusion chambers [Benayahu et al., 1989] or by implanting these cells at ectopic sites [Benayahu et al., 1994a]. Herein, we focused on identifying the short-term modulation of intracellular Ca^{+2} and gap-junction communication following estrogen-derivatives treatment in vitro on these cell types.

MATERIALS AND METHODS

Cell Tissue Culture

Primary cultures of calvaria-derived cells were harvested from newborn rats (from Tel Aviv University under the animal welfare assurance no. A501001). The cells were prepared by an isolation technique based on the ability of bone cells to migrate from bone tissue onto glass. As previously described [Jones and Boyde, 1977], stripped parietal bones were fragmented and transferred onto glass cover slips (either 22 x 22 or 10.5 x 22 mm), which were placed in a 35-mm petri dish (Falcon, New Jersey) containing 2 ml of Minimum Essential Medium (MEM) (Biological Industries, Bet-Haemek, Israel) supplemented with 10% fetal calf serum (FCS) (Biological Industries). After 5 to 7 days the bone fragments were surrounded by an extended outgrowth zone of cells. At this phase of growth, the cells were in contact with each other but did not reach a complete confluent state. Bone marrow-derived stromal osteoblastic cell line, MBA-15 [Benayahu et al., 1989], was seeded on tissue culture plates in a high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS.

Stock cultures were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air. The cell culture medium was replaced every 3 days. For experiments, the cells were investigated for their response to hormones when they were in contact with each other, but did not reach complete confluence.

Inducers Used in Vitro

The estrogen derivatives used—17α-estradiol, 17β-estradiol, β-estradiol-6-(O-carboxymethyl) oxime (estradiol-CMO), and Tamoxifen (Sigma, St. Louis, MO)—were prepared by dissolving in ethanol and the stock was kept at -20°C. The final concentration of ethanol never exceeded 0.01%. This ethanol concentration was without effect on intracellular calcium concentration. 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma) was dissolved in distilled water.

Measurement of Cytoplasmic Free Ca^{+2}

Cytoplasm free Ca^{+2} was monitored using the calcium-sensitive fluorescent probe, Fura2-AM [Grynkiewicz et al., 1985]. Cells on a cover glass were loaded with 2 μM Fura2-AM (Molecular Probes, Inc., Eugene, OR) for 30 min at 37°C. The cells were washed twice in a 4-(2-hydroxyethl)-1-piperazineethanesulfonic acid (Hepes) buffer containing NaCl 145 mM; KCl 5 mM; $CaCl_2$ 2 mM; $MgCl_2$ 1 mM; glucose 10 mM; Hepes 10 mM, pH 7.3. The measurement was carried out in a 10 x 10 mm rectangular cuvette that contained a triangular adaptor to which a monolayer of bone cells grown on a coverglass (10.5 x 22 mm) was introduced. Ratio mode fluorescence of Fura2-AM (excitation 340 and 380 nm; emission 510 nm; slits 4 nm) was monitored by a SLM 8000 spectrofluorimeter and data was recorded and analyzed on a PC. Fluorescence background, under the conditions

of Fura2-AM experiments, was less than 5%. Calibration of the Fura2-AM signal was performed by methods described for Quin-2. Briefly, the cells were lysed with digitonin (25 μg/ml) to obtain maximum fluorescence. Next, 10 mM EGTA and NaOH, sufficient to elevate the pH to 8.5, were added to obtain the minimum fluorescence. Calculation of Ca^{+2} was performed as described by Grynkiewicz et al. [Grynkiewicz et al., 1985].

Measurement of Intercellular Communication

The intercellular communication via gap junctions was measured by a method based on the microinjection of a fluorescent dye, Lucifer Yellow CH (Sigma) into a single cell in a cell monolayer by a conventional micropipette (non polished). Micropipettes were filled at their tips with 4% (w/v) aqueous Lucifer Yellow CH [Stewart, 1978]. The cultured cells grown on the coverslip (22 x 22 mm) were transferred to the experimental chamber and perfused with 4-(2-hydroxyethl)-1-piperazineethanesulfonic acid (Hepes) buffer containing NaCl 145 mM; KCl 5 mM; $CaCl_2$ 2 mM; $MgCl_2$ 1 mM; glucose 10 mM; Hepes 10 mM, pH 7.3. After cell impalement by a microelectrode, hyperpolarizing pulses (20 nA) were applied for 20 s in order to eject the dye from the pipette into the cell. Spreading of the dye from the impaled cell into its neighboring cells was assayed, under epifluorescence illumination, by counting the number of fluorescent cells coupled to the microinjected cell within 2 min following the voltage pulses. The results are given in terms of mean ± SEM (n indicates the number of independent microinjection experiments).

Statistical Analysis

The data were summarized as standard error of the mean (S.E.M), after being analyzed using double-sided *t*-test to study the significance of the differences in the number of coupled cells.

RESULTS

Effect of Estrogen on Intracellular Calcium Levels

The basal level of intracellular calcium concentration measured 135 ± 8 nM (n = 21) in primary calvaria cells (Fig. 1A,B) and ranged between 154 and 134 ± 12 nM (n = 21) in MBA-15 cells (Fig. 1C,D). Following the exposure of the primary calvaria cells to 100pM 17(β-estradiol, the level of $[Ca^{+2}]_i$ increased to 235 ± 6 nM (n = 18), after a lag period of 25 ± 3 s (n = 21) (Fig. 1A)). Similar exposure of MBA-15 cells resulted in, after a lag time of 20 ± 2 s, an increased $[Ca^{+2}]_i$ to 282 ± 8 nM (n = 16) (Fig. 1C). $[Ca^{+2}]_i$ dropped rapidly after approximately 80 s to a constant level of 172 ± 6 nM (n = 18) in the primary cells. In MBA-15 cells, $[Ca^{+2}]_i$ decreased after about 70 s to a level of 216 ± 8 nM (n = 16). For both cell types, the final $[Ca^{+2}]_i$ levels were higher than their initial basal levels ($P < 0.05$). The addition of 17β-esradiol was without effect at concentrations below 1 pM or above 1 nM (data not shown). However, between these two concentration, the intracellular calcium concentration possessed a bell-shaped dependence on estradiol concentration, with maximal activity at 100 pM 17β-estradiol for both cell types (Fig. 2).

Estradiol-CMO induced increased $[Ca^{+2}]_i$ after a lag time of 15 ± 3 s in both cell types (Fig. 1B,D). The level of $[Ca^{+2}]_i$ increase, following estradiol-CMO (Fig. 1B,D), was lower then the effect of 17β-Estradiol (Fig. 1A,C) in both cell types. The dose response of $[Ca^{+2}]_i$ to estradiol-CMO shows maximal activity at 1 nM for both cells (Fig. 2). The increased levels of $[Ca^{+2}]_i$ achieved after exposure to serial dilution of estradiol-CMO, was lower than when exposed to the same concentration range of 17β-estradiol (Fig. 2). From these results it also seems that 17β-estradiol is tenfold more potent than estradiol-CMO. 17α-estradiol had no effect on the level of $[Ca^{+2}]_i$ basal at the concentration range of 10 μM to 10 M (Fig. 2).

When cells were preincubated for 45 min with the antiestrogen tamoxifen at two concentrations, 10 nM and 1 μM, followed by the addition of 100 pM 17β-estradiol, there was no modification of the basal $[Ca^{+2}]_i$ and no inhibition of the effects of 17β-estradiol on intracellular calcium.

Effect of Estrogen on Cell-to-Cell Communication

Primary calvaria cells and MBA-15 stromal osteoblastic cells were incubated with 100 pM 17α-estradiol, 100 pM 17β-estradiol, 1 nM estradiol-CMO, and 10 nM tamoxifen for different periods of time. Microinjection of the Lucifer Yellow (LY) into a single cell of the primary calvarial monolayer of cells resulted in 35 ± 4 (n = 56) neighboring coupled cells within 2 min after dye injection (Fig. 3A). The MBA-15 cells

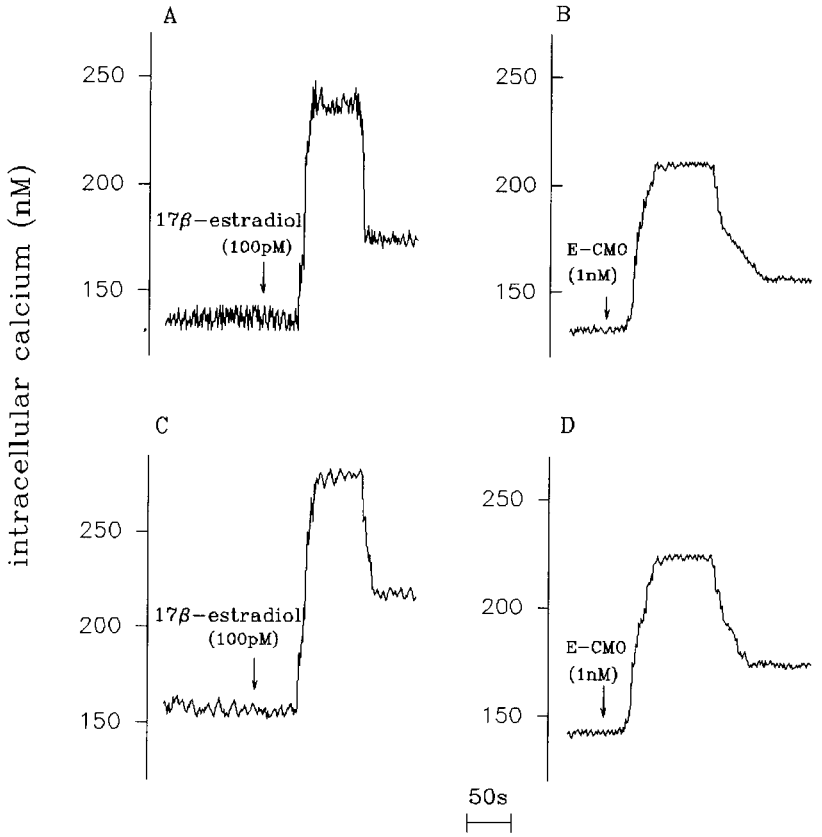

Fig. 1. Intracellular calcium levels in confluent osteoblasts were measured in cultured cells. **A,B:** Primary calvarial bone cells. **C,D:** MBA-15 marrow stromal osteoblasts. Cell response to 100 pM 17β-estradiol (A,C) or 1 nM estradiol-CMO (B,D) was measured by quick changes of $[Ca^{+2}]_i$ in s. There was an increase in $[Ca^{+2}]_i$ levels that was then lowered.

possessed 32 ± 3 (n = 61) neighboring coupled cells (Fig. 3B). Ten minutes after incubation with 17β-estradiol (100 pM) the number of coupled cells dropped down to 22 ± 3 cells in primary calvaria cells (Fig. 3A) and to 30 ± 4 coupled cells in the MBA-15 cells (Fig. 3B). Nearly full uncoupling was observed after 30 min in primary calvaria cells (Fig. 3A) and after 40 min in the MBA-15 cells (Fig. 3B). However, the uncoupling effect started to decrease after 50 min and extensive coupling was achieved after 70 min in both cultures. In primary calvarial cells, the coupling level was 90% relative to the initial coupling level and MBA-15 cells reached only 60% of their initial coupling in the same time period.

Incubation with estradiol-CMO caused a steeper decrease of coupling in primary calvarial cells (Fig. 3A), whereas the MBA-15 cells showed a slower decrease of uncoupling (Fig. 3B). Full uncoupling was seen in primary cultures after 20 min (Fig. 3A) and in MBA-15 after 30 min (Fig. 3B). The uncoupling effect lasted for only 10 min in both cultures, before cells regained their coupling after 70 min. Concentrations higher than 100 pM and 1 nM for 17β-estradiol and 1 nM estradiol-CMO, respectively, had no effect on intercellular coupling. Additionally, 17α-estradiol and the antiestrogen, tamoxifen, had no effect on the number of coupled cells (Fig. 3).

In order to understand the underlying mechanism of estrogen effect on cell-cell coupling, and based on the proposed idea that activation of PKC is involved in the coupling process in different cell types, we examined the hormonal stimulation in the presence of 50 nM TPA. Incubation of primary calvaria cells with TPA led to cell uncoupling after 40–60 min. In this case, the cells started to regain intercellular coupling (11 ± 4 coupled cells) after 2 h. The incubation of cells with estrogen (17β-estradiol or E-CMO derivatives) in the presence of TPA led to a more rapid (5–15 min) uncoupling as

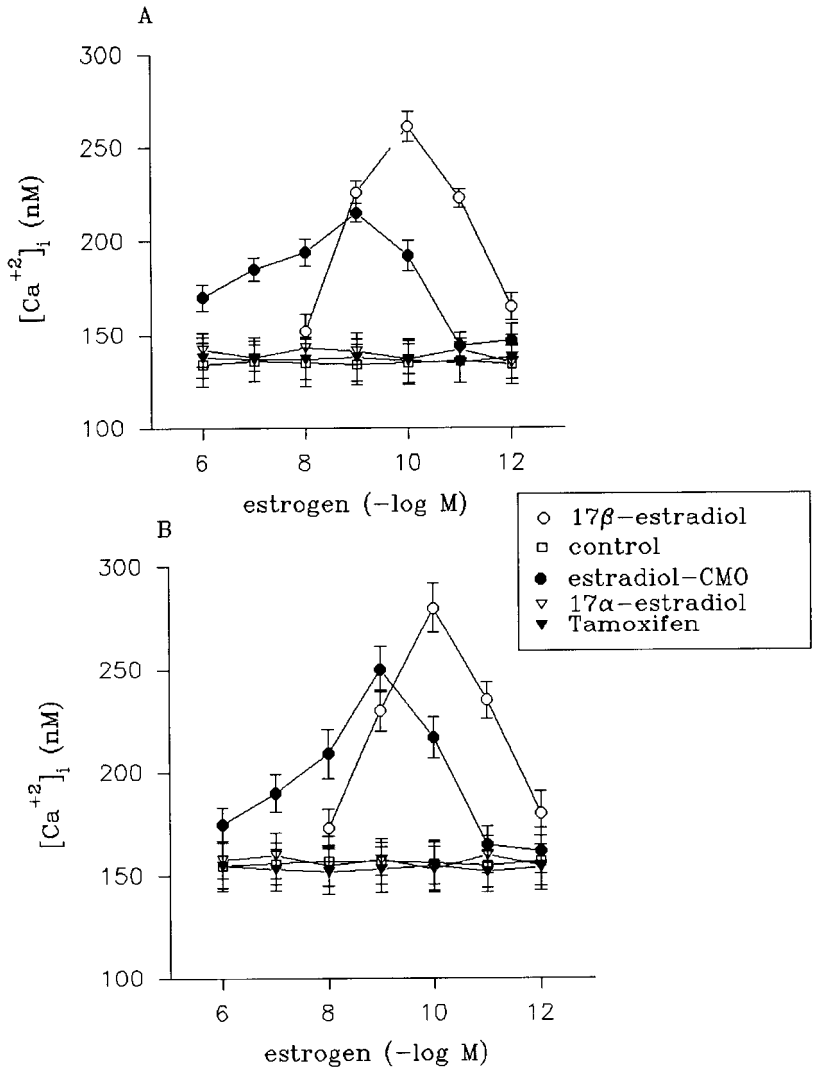

Fig. 2. Dose-dependent effects of estrogen derivatives on $[Ca^{+2}]_i$ in both **(A)** primary calvaria cells and **(B)** marrow stromal MBA-15 osteoblasts cells were determined by incubation with various concentrations 10^{-6} to 10^{-12} of 17β-estradiol, estradiol-CMO, 17α-estradiol, and tamoxifene. The elevation of intercellular $[Ca^{+2}]_i$ was achieved in a dose-dependent concentration. Values are given as a mean ± SEM of 24 cells for each steroid.

compared with the uncoupling process following incubation with TPA alone (40–60 min) and that observed upon exposure with either of these compounds (Table I). However, a long exposure (overnight) to TPA caused full restoration of cell coupling. At this stage, an addition of dose of TPA or 17β-estradiol was ineffective.

DISCUSSION

The osteoblastic cellular responses to estrogen derivatives were measured by a fast rise in the free intracellular calcium concentration. Similar results were previously reported for primary calvarial bone cells [Lieberherr et al., 1993]. Here we were able to also demonstrate the response by MBA-15 cells. The effect of 17β-estradiol is highly stereospecific since 17α-estradiol has no effect on these osteoblastic cells. Previously we were able to demonstrate nuclear receptor binding of 17β-estradiol in MBA-15 cells by in situ hybridization and by receptor binding assay, as well as, functional modulation of these cells in vitro [Shamay et al., 1996]. The MBA-15 cells' growth was inhibited by 50% in the presence of 17β-estradiol compared to cells that grew in basal medium without the hormone. An inhibiting effect of estrogen on cell proliferation was also monitored in UMR-106 cells [Gray et al., 1987, 1989]. However, these results were in contrast to the

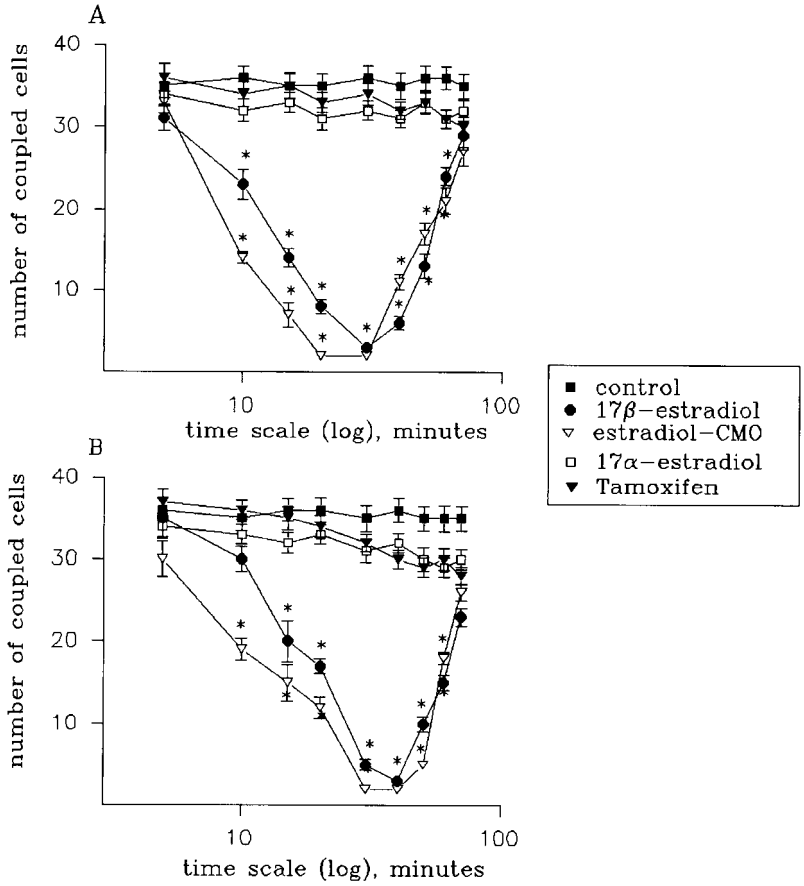

Fig. 3. Time-dependent effects of estrogen derivatives on gap-junction communication between **(A)** calvaria cells and **(B)** MBA-15 osteoblastic cell line. Cells were preincubated with different steroids (10^{-10}M 17β-estradiol, 10^{-9}M estradiol-CMO, 10^{-10}M 17α-estradiol) and with an antiestrogen (10^{-8}M tamoxifen) for periods of 5 to 70 min. Following each preincubation, the intercellular coupling was determined, and the results are expressed as mean ± SEM of 55–65 microinjections for each steroid (*$P < 0.001$ relative to the basal level).

effect of estrogen on SaOS-2 human osteosarcoma cell proliferation [Slootweg et al., 1992] and on immortalized clonal rat osteoblastic lines, RCT-1 and RCT-3 [Ernst et al., 1989]. Functional modulation of the estrogen effects on enzymatic activities of CD10/NEP and alkaline-phosphatase was studied in the MBA-15 cells. Both are enzymes that are associated with osteoblastic cells [Indig et al., 1990] and were shown to be upregulated under estrogen treatment in a dose-dependent manner [Shamay et al., 1996] similarly to the increased ALK-P activity in UMR-106 cells [Gray et al., 1987]. Other studies have shown effects on other osteoblastic functions such as an increase in the expression of mRNA for type I procollagen and elevated production of IGF binding protein [Ernst et al., 1989; Schmid et al., 1989]. In SaOS-2 human osteosarcoma cells, the estrogen treatment decreased PTH-stimulated adenylate cyclase activity [Fukayama and Tashjian, 1989]. The variation in the cells' response depends on their origin and location in vivo, as well as on their stage of differentiation.

In this study, we further characterize the cells' responses to estrogen by fast changes in intracellular calcium. The stereospecificity of these responses seems to suggest the involvement of receptors at the membrane level in transducing the estrogen's effect, rather than the participation of the established nuclear receptors in the process. The existence of estrogen membrane receptor was demonstrated on various types of cells [Pappas et al., 1994, 1995]. This is further supported by the ineffectiveness of tamoxifen, which competes with 17β-estradiol or estradiol-CMO for nuclear receptors [Ernst et al., 1988], in abolishing the estrogen-induced elevation of intracellular calcium or its uncoupling effect. It has been shown that other

TABLE I. Estrogen Effect on Osteoblast Cell Coupling*

	Primary calvarial cells	Marrow stromal, MBA-15
Control		
Basal medium	Coupled	Coupled
Group A		
17β-estradiol (100 pM)	Uncoupled (30 min)	Uncoupled (40 min)
Estradiol-CMO (1 nM)	Uncoupled (20–30 min)	Uncoupled (30–40 min)
TPA (50 nM)	Uncoupled (40–60 min)	Uncoupled (60–65 min)
Group B		
TPA (50 nM) + 17β-estradiol (100 pM)	Uncoupled (10–15 min)	Uncoupled (5–10 min)
TPA (50 nM) + estradiol-CMO (1 nM)	Uncoupled (10–15 min)	Uncoupled (5–10 min)
TPA (50 nM) + 17α-estradiol (100 pM)	Uncoupled (40–60 min)	Uncoupled (60–70 min)
TPA (50 nM) + tamoxifen (1 nM)	Uncoupled (40–60 min)	Uncoupled (60–70 min)

*Measurement of number of cells coupling following their exposure. Control group are cells in basal medium. The number of coupled primary calvarial cells was (35 ± 6); the number of marrow stromal, MBA-15 cells was (32 ± 6). Time for coupling measurement was 2 min following microinjection. Group A, determines the uncoupling time of exposured cells to various hormones as detailed; Group B, combined exposure to 50 nM TPA and the different hormones.

steroids (like calcitriol, the hormonally active form of vitamin D) and estrogens increase intracellular calcium concentration in osteoblasts [Lieberherr et al., 1993; Civitelli et al., 1990] and in other cell types [Lieberherr et al., 1989; Bourdeau et al., 1990; Wali et al., 1990]. The 17β-estradiol-induced elevation of $[Ca^{+2}]_i$ shows a bell-shaped dependence, with maximal activity at 100 pM. This bell-shaped profile has also been found for the genomic action of 17β-estradiol on osteoblasts [Ernst et al., 1988]. A similar effect was found for non-genomic response of calcitriol in different cell types [Lieberherr et al., 1989; Bourdeau et al., 1990]. The elevation of intracellular $[Ca^{+2}]_i$ by 17β-estradiol occurs through Ca^{+2} influx from the extracellular milieu and mobilization of Ca^{+2} from the endoplasm reticulum in the cells. This is based on the finding that estrogens cause alterations of membrane phospholipid turnover in osteoblsats by formation of inositol 1,4,5-trisphosphate (IP_3) with the consequent released Ca^{+2} from intracellular pools and formation of diacylglycerol (DAG) with successive activation of protein kinase C (PKC) [Lieberherr et al., 1993; Migliaccio et al., 1993]. This PKC activation was shown to lead to an inhibition of intercellular communication [Enomoto and Yamasaki, 1985; Gainer and Murray, 1985]. We further examined the effect of estrogens on cell-cell communication. Indeed, incubation of primary calvarial cells and MBA-15 osteoblastic cell line with estrogens caused a gradual time-dependent decrease of inter-cellular communication. Full uncoupling was reached after 20–40 min followed by recovery of coupling within an additional 40 min. The simultaneous exposure of bone cells to TPA and estrogen caused uncoupling within a shorter time period. It was suggested that uncoupling proceeded through the activation of PKC following preincubating the cells with TPA [Chida et al., 1986]. The rapid inhibition of gap junction permeability, in the combined presence of TPA and estrogen, may be correlated with a faster translocation of PKC from cytosolic to membrane compartments with concomitant phosphorylation of the gap-junction proteins. The swift recoupling stage, following uncoupling, may be attributed either to a fast PKC down-regulation or to the increase of phosphatase activity, or both.

Estrogen is believed to play an important role in bone metabolism and bone mass levels in the adult life, by maintaining a balance between osteoblastic and osteoclastic activity. When estrogen is deficient, there is evidence for an increase in activation frequency of bone remodeling and an increase in resorption that leads to an imbalance in bone formation. This may be due to the modulation of metabolic cooperativity in bone cells, which provides a coordinated regulation of bone formation. The present study suggests that the modulation of gap junction communication and intracellular $[Ca^{+2}]_i$ levels by estrogens provides an additional pathway for the effect of sex hormones that may regulate the proliferation and function of osteoblastic cells via membrane receptors.

REFERENCES

Arnet TR, Horton MA, Colston KW, Fraser DI, Taylor L, King RJB, Stevenson JC (1987): Estrogen receptors and human bone cells: An immunocytochemical study. In Christiansen C, Johansen JS, Riis BJ (eds): "Osteoporosis." Copenhagen: Osteopress, pp. 519–538.

Benayahu D, Kletter Y, Zipori D, Wientroub S (1989): Bone marrow derived stromal cell line expressing osteoblastic phenotype in vitro and osteogenic capacity in vivo. J Cell Physiol 140:1–7.

Benayahu D, Fried A, Zipori D, Wientroub S (1991): Subpopulations of marrow stromal cells share a variety of osteoblastic markers. Calcif Tissue Int 49:202–207.

Benayahu D, Gurevitch O, Zipori D, Wientroub S (1994a): Bone formation by marrow osteogenic cells (MBA-15) is not accompanied by osteoclastogenesis and generation of hematopoietic supportive microenvironment. J Bone Miner Res 9:1107–1114.

Benayahu D, Kompier R, Shamay A, Kadouri A, Zipori D, Wientroub S (1994b): Mineralization of marrow-stromal osteoblasts MBA-15 on three-dimensional carriers. Calcif Tissue Int 55:120–127.

Benayahu D, Fried A, Shamay A, Cunningham N, Blumberg S, Wientroub S (1994c): Differential effects of retinoic acid and growth factors on osteoblastic markers and CD10/NEP activity in stromal-derived osteoblasts. J Cell Biochem 56:62–73.

Benayahu D, Fried A, Efraty M, Gehron Robey P, Wientroub S (1995a): Bone marrow interface: Preferential attachment of an osteoblastic marrow stromal cell line. J Cell Biochem 58:151–160.

Benayahu D, Fried A, Wientroub S (1995b): PTH and 1,25(OH) vitamin D priming to growth factors differentially regulates the osteoblastic markers in MBA-15 clonal subpopulations. Biochem Biophys Res Commun 210:197–204.

Bingmann D, Tetsch P, Massas R (1988): Membraneigenschaften von zellen aus knochenexplanaten. Z Implantol 4:277–281.

Bourdeau A, Atmani F, Grosse B, Lieberherr M (1990): Rapid effects of 1,25-dihydroxy vitamin D_3 and extracellular Ca^{+2} on phospholipid metabolism in dispersed pocrine parathyroid cells. Endoc 127:2738–2743.

Chida K, Kato N, Kuroki T (1986): Down regulation of phorbol diester receptors by proteolytic degradation of protein kinase C in a cultured cell lines of fetal rat skin keratinocytes. J Biol Chem 262:13013–13018.

Christiansen C, Christensen MS, Transbol I (1981): Bone mass in post menopausal women after withdrawal of estrogen/progestogen replacement therapy. Lancet 2:459–461.

Civitelli R, Kim YS, Gunsten SL, Fujimori A, Huskey M, Avioli LV, Hruska KA (1990): Nongenomic activation of calcium message system by vitamin D metabolites in osteoblast-like cells. Endocrinology 127:2253–2262.

Civitelli R, Beyer EC, Warlow PM, Robertson AJ, Geist ST, Steinberg TH (1993): Connexin43 mediates direct intercellular communication in human osteoblastic cell networks. J Clin Invest 91:1888–1896.

Dahl G, Berger W (1978): Nexus formation in the myometrium during parturition and induced by estrogen. Cell Biol Int Rep 2:377–381.

De Mello WC (1988): Cyclic nucleotides and junctional permeability. In Hertzberg EL, Johnson (eds): "Gap Junctions." New York: Alan R. Liss, Inc., pp 245–254.

Doty SB (1981): Morphological evidence of gap junctions between bone cells. Calcif Tissue Int 33:509–512.

Enomoto Y, Yamasaki H (1985): Rapid inhibition of intercellular communication between BALB/C 3T3 cells by diacylglcerol a possible endogenous functional analogue of phorbol esters. Cancer Res 45:3706–3710.

Eriksen EF, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC, Riggs BL (1988): Evidence of estrogen receptors in normal human osteoblast-like cells. Science 241:84–86.

Ernst M, Schmid CH, Froesch ER (1988): Enhanced osteoblast proliferation and collagen gene expression by estradiol. Proc Natl Acad Sci USA 85:2307–2310.

Ernst M, Heat JK, Rodan GA (1989): Estradiol effect on proliferation, messenger ribonucleic acid for collagen and insulin-like growth factor-I and parathyroid hormone-stimulated adenylate cyclase activity in osteoblastic cells from calvariae and long bones. Endocrinology 125:825–833.

Fukayama S, Tashjian AH (1989): Direct modulation by estradiol of the response of human bone cells (SaOS-2) to human parathyroid hormone (PTH) and PTH-related protein. Endocrinology 124:397–401.

Gainer HStC, Murray AW (1985): Diacylglycerol inhibits gap junctional communication in cultured epidermal cells: Evidence for a role of protein kinase C. Biochem Biophys Res Commun 126:1109–1113.

Gray TK, Flynn S, Gray KM, Nabell LM (1987): 17β-Estradiol acts directly on the clonal osteoblastic cell line UMR 106. Proc Natl Acad Sci USA 84:6267–6271.

Gray TK, Mohan S, Linkhart TA, Baylink DJ (1989): Estradiol stimulates in vitro the secretion of insulin-like growth factors by the clonal osteoblastic cell line, UMR 106. Biochem Biophys Res Commun 158:407–412.

Grynkiewicz G, Poenie M, Tsien RY (1985): A new generation of Ca^{+2} indicator with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Ikegami A, Inoue S, Hosoi T, Mizuno Y, Nakamura T, Ouchi Y, Orimo H (1993): Immuno-histochemical detection and Northern blot analysis of estrogen receptor in osteoblastic cells. J Bone Miner Res 8:1103–1109.

Indig FE, Benayahu D, Fried A, Wientroub S, Blumberg S (1990): Neutral endopeptidase (EC 3.4.24.11) is highly expressed in osteoblastic cells and other marrow stromal cell types. Biochem Biophys Res Commun 172:620–626.

Ivey JL, Baylink JD (1981): Postmenopausal osteoporosis: Proposed roles of defective coupling and estrogen deficiency. Metab Bone Dis 3:3–7.

Jones SJ, Boyde A (1977): Migration of osteoblasts. Cell Tissue Res 184:179–193.

Komm BS, Terpening CM, Benz DJ, Graeme KA, Gallegos A, Korc M, Green GL, O'Mally BW, Haussler MR (1988): Estrogen binding, receptor mRNA and biological response in osteoblast-like osteosarcoma cells. Science 241: 84–86.

Lieberherr M, Grosse B, Duchambon P, Druck T (1989): A functioning cell type receptor is required for the early action of 1,25-dihydroxy vitamin D_3 on phospholipid metabolism in rat enterocytes. J Biol Chem 264:20403–20406.

Lieberherr M, Grosse B, Kachkache M, Balsan S (1993): Cell signaling and estrogens in female rat osteoblasts: A possible involvement of unconventional nonnuclear receptors. J Bone Miner Res 8:1365–1375.

Lindsay R, Hart DM, Forrest C, Baird C (1980): Prevention of spinal osteoporosis in oophorectomized women. Lancet 2:151–153.

Manolagas SC, Jilka RL (1994): Bone marrow, cytokines and bone remodeling. N Engl J Med 232:305–311.

Massas R, Bingmann D, Korenstein R, Tetsch P (1990): Membrane potential of rat calvaria bone cells: dependence on temperature. J Cell Physiol 144:1–11.

Massas R, Pitaru S, Weinreb M (1993): The effect of titanium and hydroxyapatite on osteoblastic expression and proliferation in rat partial bone cultures. J Dent Res 72:1005–1008.

Merk FB, Botticelli CR, Albright JT (1978): An intercellular response to estrogen by granulosa cells in the rat ovary; an electron microscopy study. Endocrinology 90:992–1007.

Migliaccio S, Wetsel WC, Fox WM, Washburn TF, Korach KS (1993): Endogenous protein kinase C activation in osteoblast-like cells modulates responsiveness to estrogen and estrogen receptor levels. Mol Endocrinol 7:1133–1143.

Pappas TC, Gametchu B, Yannariello-Browm J, Collins TJ, Watson CS (1994): Membrane estrogen receptors in GH3/B6 cells are associated with rapid estrogen-induced release of prolactin. Endocrine 2:813–822.

Pappas TC, Gametchu B, Watson CS (1995): Membrane estrogen receptors identifed by multiple antibody labelling and impeded ligand binding. FASEB 9:404–410.

Riggs BL, Jowsey J, Goldsmith RS, Kelly PJ, Hoffman LD, Arnaud CD (1972): Short- and long-term effects of estrogen and synthetic anabolic hormone in postmenopausal osteoporosis. J Clin Invest 51:1659–1663.

Rose B, Loewenstein WR (1975): Permeability of cell junction depends on local cytoplasmic calcium activity. Nature 254:250–252.

Saez JC, Spray DC, Nairn AC, Hertzberg E, Greengard P, Bennett MVL (1986): cAMP increases junctional conductance and stimulates phosphorylation of the 27-kDa principal gap junction ploypeptide. Proc Natl Acad Sci USA 83:2473–2477.

Schmid C, Ernst M, Zapf J, Froesch ER (1989): Release of insulin-like growth factor carrier proteins by osteoblasts: Stimulation by estradiol and growth hormone. Biochem Biophys Res Commun 160:788–794.

Shamay A, Knopov V, Benayahu D (1996): The expression of estrogen receptor and estrogen effect in MBA-15 marrow stromal osteoblasts. Cell Biol Int 20:401–405.

Shen V, Rifas L, Kohler G, Peck WA (1986): Prostaglandins change cell shape and increase intercellular gap junctions in osteoblasts cultured from rat fetal calvaria. J Bone Miner Res 1:243–249.

Slootweg MC, Ederveen AGH, Schot LPC, Schoonen WGEJ, Kloosterboer HJ (1992): Oestrogen and progestogen synergistically stimulate human and rat osteoblast proliferation. J Endocrinol 133:R5–R8.

Somogyi R, Batzer A, Kolb HA (1989): Inhibition of electrical coupling in pairs of murine pancreatic acinar cells by OAG and isolated protein kinase C. J Membr Biol 108: 273–283.

Steinberg TH, Civitelli R, Geist ST, Robertson AJ, Hick E, Veenstra RD, Wang HZ, Warlow PM, Westphale EM, Laing JG, Beyer EC (1994): Connexin43 and connexin45 form gap junctions with different molecular permeabilities in osteoblastic cells. EMBO J 13:744–750.

Stewart WW (1978): Functional connections between cells as revealed by dye-coupling with a high fluorescent naphthalimid tracer. Cell 14:741–759.

Swenson KI, Piwnica-Warms H, McNamee H, Paul DL (1990): Tyrosin phosphorylation of the gap junction protein connexin43 is required for the $pp60^{v\text{-}src}$-induced inhibition of communication. Cell Regul 1:989–1002.

Tetsch P, Massas R, Bingmann D (1989): Der einfluss von anderungen des extrazellularen milieus auf das membranpotential von zellen in der Knochenzellkultur. Z Implantol 5:52–58.

Turin L, Warner A (1977): Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryos. Nature 270:56–57.

Wali RK, Baum CL, Sitrin MD, Brasitus TA (1990): 1,25-dihydroxy vitamin D_3 stimulates membrane phosphoinositide turnover, activates protein kinase C, and increases cytosolic calcium in rat colonic epithelium. J Clin Invest 85:1296–1303.