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Simultaneous Detection and Functional Response of Testosterone and Estradiol Receptors in Osteoblast Plasma Membranes

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Abstract Osteoblasts derived from the periosteal surfaces of two–three-week-old male broiler chicken tibias were cultured for eight days. The cells were then loaded with fura-2/AM ester to detect surges in intracellular Ca^{2+}. Treatment with 10^{-7} M testosterone (T) or 17β-estradiol (E) elicited a rapid (within seconds) response that was substantially reduced by introducing the calcium chelating agent EGTA or the calcium-channel blocker verapamil. The hormones were equally effective when covalently linked to bovine serum albumin (BSA), a procedure that ensures the hormone does not enter the cells. The rapid response to surface-bound steroids indicates that the responses were invoked through plasma-membrane receptors. The source of Ca^{2+} was shown to be through entry from external sources, as well as from intracellular stores. Flow cytometry of fluorescein-tagged T-BSA and E-BSA revealed that osteoblasts derived from male chickens had similar and substantial levels of both receptors. J. Cell. Biochem. 79: 620–627, 2000.

Key words: osteoblasts; 17β-estradiol; testosterone; plasma membrane receptors

Sex steroids are profoundly important in the development and maintenance of the skeleton. For example, the development of osteoporosis, a metabolic bone disease characterized by low bone mass, is associated with decreased estrogen production at the cessation of menses. Estrogen replacement therapy has been shown to partially restore bone metabolism, to minimize bone loss, and to reduce hip and vertebral fractures [Lindsay and Cosman, 1996]. It is now well established that osteoblasts, as well as many other estrogen-responsive tissues, possess nuclear estrogen receptors [Harris et al., 1996]. Less well defined are steroid receptors found in the plasma membrane. Although the exact role of the membrane receptors remains to be clarified, a wide spectrum of cell types have been reported to contain such receptors, as reviewed recently [Brubaker and Gay, 1999]. Cell types include, for example, rat uterine cells [Pietras and Szego, 1979], rat pituitary cells [Bression et al., 1986], MCF-7 and MDA-MB-231 human breast cancer cell lines [Benz et al., 1985], avian osteoclasts [Brubaker and Gay, 1994], and primary calvarial neonate rat osteoblasts [Lieberherr et al., 1993; Massas et al., 1998].

Testosterone, like estradiol, has also been implicated as a major regulator of bone metabolism [Crilly et al., 1981; Orwoll, 1996]. Hypogonadism in males, which results from decreased levels of testosterone, is associated with low bone mass and osteoporosis; treatment of this condition with androgens results in a reversal of bone loss and a lowering of associated risk of fracture. Further, testosterone deficiency has been implicated as a causative factor in female osteoporosis with the decline of adrenal androgens associated with aging [Gasperino, 1995; Wild et al., 1987]. Androgens have been shown to maintain normal bone mass despite undetectable levels of estradiol [Dixon et al., 1989], and androgen therapy in postmenopausal women has been shown to prevent bone loss [Need et al., 1989]. Androgen

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receptors have been identified in the nucleus and cytosol of osteoblasts [Nakano et al., 1994; Orwoll et al., 1991]. Furthermore, it has been shown that androgen receptors are predominantly expressed in osteoblasts at sites of bone formation in human bone, with the pattern and number of cells expressing the receptor being similar in both sexes [Abu et al., 1997]. The presence of a testosterone plasma membrane receptor has also been reported in, for example, rat liver and prostate cells [Konplya and Popoff, 1992], mouse T-cells [Benten et al., 1999], osteosarcoma cells [Takeuchi and Guggino, 1996], and male rat osteoblasts [Lieberherr and Grosse, 1994]. In the latter three studies, receptor occupancy has been shown to elicit intracellular calcium increases through ion-mediated channels.

Our study focuses on demonstrating the existence of functional testosterone and estradiol receptors simultaneously in the plasma membrane of primary osteoblasts derived from one gender. To accomplish this, the action of the steroid in causing a calcium influx was investigated and characterized in osteoblasts, derived from a male animal model, through fluorescence spectroscopy. Flow cytometry was used to compare fluorescent labeling of the two plasma membrane hormone receptors and shows that both testosterone and estradiol receptors exist simultaneously in osteoblast plasma membrane, independent of gender.

MATERIALS AND METHODS

Materials

HEPES buffer, Dulbecco's modified Eagle's medium D2902 (DME), minimal essential medium Eagle's M3024 (MEM), collagenase (type IA), fetal bovine serum (FBS), 0.25% typsin-0.02% EDTA solution, bovine serum albumin (BSA), β-cyclodextrin, water-soluble testosterone (testosterone encapsulated by β-cyclodextrin), water-soluble 17β-estradiol (17β-estradiol encapsulated by β-cyclodextrin), 17β-estradiol-6-carboxymethyloxime-BSA (E-BSA; 20 mol estradiol per mol BSA), EGTA, verapamil, testosterone 3-(O-carboxymethyl)-oxime-BSA (T-BSA; 26 mol testosterone per mol BSA), T-BSA-fluorescein isothiocyanate (T-BSA-FITC; 10 mol testosterone, 3 mol FITC per mol BSA), and E-BSA-FITC (10 mol estradiol, 3 mol FITC per mol BSA) were obtained from Sigma (St. Louis, MO). Nile Red was from Polysciences Inc. (Warrington, PA) and fibronectin was from Biomedical Technologies (Stoughton, MA). Trypsin (bovine pancreatic, 3× crystallized) was purchased from Worthington Biochemicals (Freehold, NJ). Pluronic, digitonin, and fura-2/acetoxymethyl-ester (fura-2/AM) were obtained from Molecular Probes (Eugene, OR).

Osteoblast Isolation

Osteoblasts were isolated from the tibia of two–three-week-old male chickens obtained from the Poultry Education and Research Center of Penn State University, as previously described [Gay et al., 1994]. Briefly, this was accomplished through mild sequential enzymatic digestion with 0.03% trypsin and 0.1% collagenase (type IA), followed by scraping with a curette. Osteoblasts were cultured on fibronectin-coated rectangular glass coverslips (22 mm × 9 mm) or on 60-mm fibronectin-coated polystyrene culture plates (Corning Glass Works, Corning, NY) in phenol red-free DME medium, supplemented with 3.7 g/l sodium bicarbonate, 0.05 g/l ascorbate, 100 U penicillin, 100 μg streptomycin, and 10% heat-inactivated FBS. Fibronectin was selected as a substrate, rather than collagen I or uncoated glass, because we found that the cultures reached confluency and began to make their own collagen I matrix more quickly. The pH indicator phenol red was omitted, due to its mild estrogenic properties [Berthois et al., 1986]. Cells were cultured for seven days.

Intracellular Calcium Measurement

The cells cultured on coverslips were rinsed three times for approximately 1 min each in 2 ml Hank's Buffer, pH 7.4 (137 mM NaCl, 0.441 mM KH_2PO_4, 0.442 mM Na_2HPO_4, 0.885 mM $MgSO_4$ - $7H_2O$, 27.7 mM glucose, 1.25 mM $CaCl_2$, 20 mM HEPES buffer, and 1mg/ml BSA). Rinsed cells were then placed in 1μM fura-2/AM in 2 ml Hank's Buffer with 20% pluronic, a detergent, at room temperature for 40 min. After loading with fura-2/AM, the cells were rinsed for 1 min in approximately 2 ml Hank's buffer. A cell-laden coverslip was then mounted into a coverslip holder (Hitachi) and inserted into a quartz (10 mm × 45 mm) cuvette that contained 1.5 ml Hank's buffer. The cuvette was then placed in the light path of a Hitachi F-2000 Fluorescence Spectrophotometer (Tokyo, Japan) and reagents were added to the cuvette under continuous stirring.

The intracellular calcium concentration ($[Ca^{2+}]_i$), measured by the fura-2 fluorescence response, was calibrated using the ratio of 340/380 nm fluorescence values after subtraction of background fluorescence at both 340 and 380 nm. Fluorescence intensity at 224 nm was used to determine the dissociation constant for the fura-2–Ca^{2+} complex, as described by Grynkiewicz, et al. [1985]. The R_{max} and R_{min} values were found through measurements in the presence and absence of 25 μM digitonin and 4 mM EGTA, and enough Tris-base to raise the pH to 8.3 or higher, as described by Lieberherr et al. [1993].

Water-soluble testosterone (10^{-7} M) and water-soluble 17β-estradiol (10^{-7} M) were introduced into the cuvette at times shown in Fig. 1. This dose was selected on the basis of earlier studies [Lieberherr et al., 1993; Lieberherr and Grosse, 1994]. Because the steroids were rendered water soluble by encapsulation in β-cyclodextrin, β-cyclodextrin (10^{-7} M) in vehicle (Hank's buffer) was used as a negative control. Next, to determine whether the effects were due to an influx of extracellular Ca^{2+}, cells were pretreated with EGTA (2 mM) or with the voltage-dependent calcium channel blocker verapamil (1 μM) 1 min prior to introducing a steroid. To ascertain that the observed rapid response to steroid treatment was due to a membrane receptor, the initial experiment was repeated with each steroid conjugated to BSA. When steroids were covalently bound to BSA, entry of the ligands into the cell and subsequent interaction with steroid receptors in the cytosol and/or nucleus was prevented. The negative control or vehicle used for evaluating conjugated steroid effects was 0.01% ethanol in Hank's buffer. The *t*-test was used to compare the Ca^{++} signal in hormone treatments versus vehicle controls (Table I).

Flow Cytometry

Cell suspensions were made by releasing cells cultured on plastic petri dishes by digestion with trypsin-EDTA solution (0.5 ml/60 mm dish) and gentle scraping. After 5 min of incubation at 37°C, six volumes of DME plus 10% FBS were added to inhibit digestion. The cells in suspension were allowed to recover at 37°C for 1 h before being incubated for 5 min with either T-BSA-FITC (10^{-6} M), E-BSA-FITC (10^{-6} M), or BSA-FITC (10^{-6} M), all dissolved in Hank's Buffer. The cells were then centrifuged at 1,800 rpm for 5 min at 4°C, the supernate discarded, and the cells resuspended in 0.5 ml MEM plus 10% FBS. A Coulter XL-MCL flow cytometer (Fullerton, CA) was used to detect the amount of fluorescence on each cell. Cells were distinguished from bone particles by Nile Red (10 ng/ml) staining. Only material that fluoresced in the Nile Red emission range (≈636 nm) and had an appropriate size were evaluated. To evaluate possible damage by trypsinization resulting in plasma membrane permeabilization, cells released by the trypsinization process were placed in 10^{-6} M BSA-FITC and examined by confocal microscopy at 5 and 10 min. The BSA-FITC complex did not enter the cells, except in rare cases (<1%) where propidium iodide staining (2.5 μg/ml) revealed that cells were not viable.

RESULTS

Effect of Testosterone and 17β-Estradiol on $[Ca^{2+}]_i$

The effects of testosterone and 17β-estradiol on intracellular calcium levels ($[Ca^{2+}]_i$) was investigated using the fura-2/AM probe for intracellular calcium in fluorescence spectrophotometry. As shown in Fig. 1A, the cells were first exposed to vehicle (β-cyclodextrin in Hank's buffer, 10^{-7} M) as a control, followed by 10^{-7} M testosterone in β-cyclodextrin in buffer at approximately 100 s. Hormone treatment initiated an increase in $[Ca^{2+}]_i$ that leveled off into a plateau, typically between 150 and 200 s after treatment. Subsequent treatment with EGTA resulted in a rapid decrease to or under original baseline levels. A second bolus of testosterone (10^{-7} M) was then introduced; this resulted in a small increase in $[Ca^{2+}]_i$ and a return to baseline levels after about 100 s. Figure 1B shows the results when 10^{-7} M 17β-estradiol was used as the stimulating agent, followed by EGTA and retreatment with a second bolus of estradiol. The average increase for each treatment, in all samples tested, was then calculated as the percent increase from baseline in $[Ca^{2+}]_i$ and is presented in Table I. Testosterone treatment resulted in a 133 ± 15% average increase, 17β-estradiol showed a 118 ± 18% average increase, whereas the control, β-cyclodextrin in vehicle, showed only a 19 ± 2% average increase. The increase due to β-cyclodextrin alone represents the maximum increase for vehicle under any experiment be-

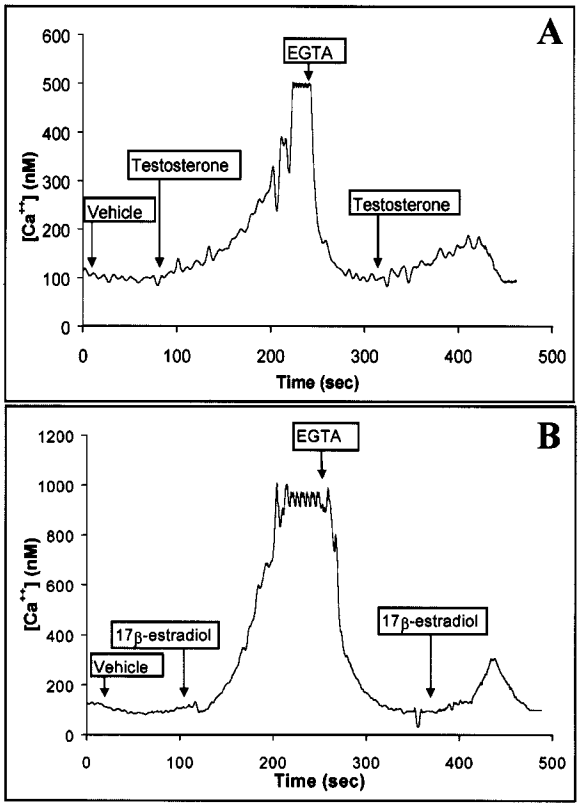


Fig. 1. Representative graph showing the effect of testosterone (**A**) or 17β-estradiol (**B**) on $[Ca^{2+}]_i$ both before and after EGTA treatment of cultured, primary osteoblasts. $[Ca^{2+}]_i$ was monitored continuously in fura-2/AM–treated cells while treatments were added under continuous stirring. Successive treatments were: β-cyclodextrin in vehicle (10^{-7} M), testosterone or 17β-estradiol (10^{-7} M), EGTA (2 mM) to bind free Ca^{2+}, and a second bolus of testosterone or 17β-estradiol (10^{-7} M). For both steroids, similar results were obtained for three replicates from five separate cell isolations.

cause intracellular calcium levels were under no other influence during this treatment. This level is taken as the negative control for all experiments using the water-soluble hormones. Following EGTA treatment, a second bolus of testosterone or 17β-estradiol elicited an increase in $[Ca^{2+}]_i$ by 40 ± 8% or 40 ± 6%, respectively, as shown in Table I.

Effect of Testosterone and 17β-Estradiol on $[Ca^{2+}]_i$ in Verapamil-Pretreated Cells

To gain insight into the source of cytosolic Ca^{2+} observed in the first set of experiments, the experiments were repeated on cells pretreated with the calcium channel blocker verapamil (1 μM). Both testosterone and 17β-estradiol stimulated a Ca^{2+} response, but then

TABLE I. Average Increase Above Baseline of Intracellular Calcium Following Treatment with Testosterone (T) and 17β-Estradiol (E) in the Presence and Absence of EGTA and Verapamil[a]

Treatment	T	E	V	n[b]
T or E[c]	133 ± 15	118 ± 18	19 ± 2	15
EGTA (2 mM), then T or E	40 ± 8	40 ± 6	—[d]	15
Verapamil (1 mM), then T or E	53 ± 15	93 ± 7	—	9
Verapamil + EGTA, then T or E	nd[e]	6.3 ± 2	—	9
T- or E-BSA[f]	102 ± 39	136 ± 79	5 ± 2	9

[a]All data represent $[Ca^{2+}]_i$ 1 min after each treatment was introduced; derived from curves such as shown in Figs. 1 and 2.
[b]n = 9 or 15; each experiment performed in triplicate on three or five separate osteoblast populations.
[c]T and E are β-cyclodextrin-encapsulated testosterone (10^{-7} M) and 17β-estradiol (10^{-7} M) in Hank's buffer; V is vehicle (Hank's buffer) plus β-cyclodextrin ($P \geq 0.1$ for T versus V and E versus V).
[d]—Not measured.
[e]No response detected.
[f]T- and E-conjugated to BSA; vehicle was BSA in Hank's buffer ($P \geq 0.1$ for T versus V and E versus V).

$[Ca^{2+}]_i$ gradually declined to baseline without development of a plateau (Fig. 2). After EGTA treatment in the verapamil-treated cells, testosterone and 17β-estradiol both caused very little or no increase in $[Ca^{2+}]_i$. As with the first set of experiments, the average increase in $[Ca^{2+}]_i$ was calculated for each treatment (Table I). Testosterone elicited a 53 ± 15% average increase before and 0% average increase after EGTA treatment; 17β-estradiol showed a 93 ± 7% average increase before and a 6 ± 2% average increase after EGTA treatment.

Effect of Testosterone and 17β-Estradiol Covalently Linked to BSA on $[Ca^{2+}]_i$

Because both testosterone and 17β-estradiol had shown the ability to elicit a $[Ca^{2+}]_i$ influx within several seconds of treatment, reaching a maximum within 200 s, the interaction of the hormones with plasma membrane receptors was implicated. To further support this claim, hormone linked to the protein BSA was administered to prevent entry into the cells. As expected, BSA in vehicle caused very little or no

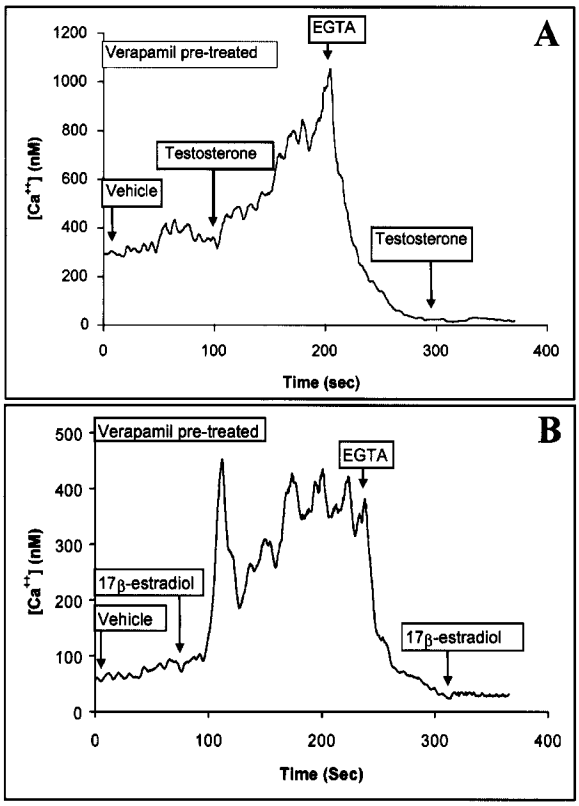


Fig. 2. Influence of verapamil on $[Ca^{2+}]_i$ in testosterone- (**A**) or 17β-estradiol- (**B**) treated osteoblasts. The cells were pretreated with verapamil (1 mM) for 1 min, treated with vehicle, testosterone or 17β-estradiol, EGTA, and, finally, a second bolus of hormone, as in Figure 1.

increase in $[Ca^{2+}]_i$. Both T-BSA and E-BSA elicited rapid increases in $[Ca^{2+}]_i$, similar to those when hormone that was not conjugated to protein (Table I). The average increase in $[Ca^{2+}]_i$ from baseline was calculated for each treatment and was 102 ± 39%, 136 ± 79%, and 5 ± 2% for T-BSA, E-BSA, and BSA alone.

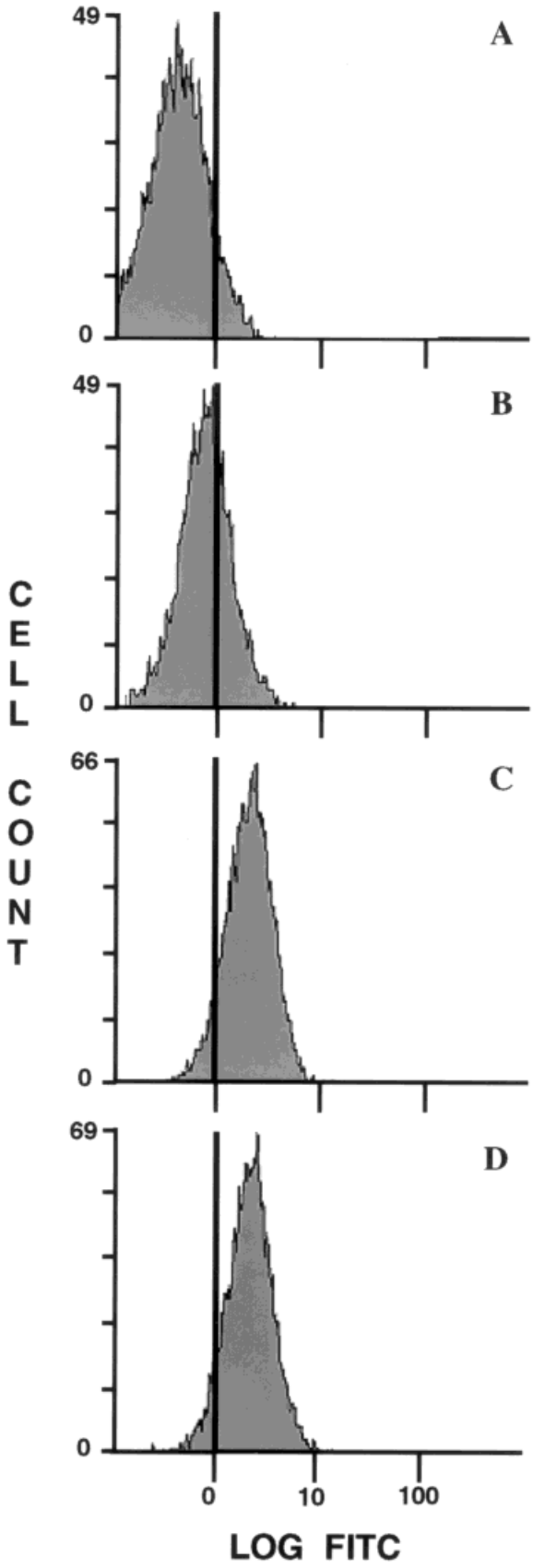
Flow Cytometry Profiles of Steroid Binding

Cell suspensions of osteoblasts from male chickens were prepared, divided into 0.5-ml aliquots and treated with either T-BSA-FITC (10^{-6} M), E-BSA-FITC (10^{-6} M), BSA-FITC (10^{-6} M), or vehicle only. The samples were then centrifuged, the supernate discarded, and the cells resuspended in MEM plus 10% FBS. For flow cytometry analysis, fluorescence intensity was recorded for 10,000 cells per sample. Three samples for each treatment were analyzed for three different cell isolations. A representative graph of the log of FITC fluorescence intensity as a function of cell number for each treatment was obtained (Fig. 3). When no treatment was applied, a very low level of fluorescence (autofluorescence) was detected for the cell population (Fig. 3A). When cells exposed to BSA-FITC were introduced, a small shift in fluorescence, which centers to the left of the vertical line (Fig. 3B), resulted. Figure 3C and 3D show the results of treating with T-BSA-FITC and E-BSA-FITC, respectively. Both of these treatments show a shift to higher fluorescence by an order of magnitude, with the cell population peaks lying to the right of the vertical line. The area under the peak on these graphs represents all 10,000 cells counted by the cytometer except for a small number (<100) that show no fluorescence. Peak height, peak area, and peak shift were similar for both T-BSA and E-BSA.

DISCUSSION

This study corroborates earlier studies showing that plasma membrane receptors for testosterone and estrogen are present in primary osteoblasts [Lieberherr et al., 1993; Lieberherr and Grosse, 1994; Massas et al., 1998]. Such corroboration is important because plasma membrane receptors for steroids is still a controversial issue. The study broadens the knowledge base by using cells derived from a different source than previously reported. These differences include: species (avian versus mammal), bone type (long bone versus calvarial, appendicular versus axial, endochondral versus intramembrous) and age (three weeks of age in a rapidly growing species versus neonate of a moderate growth-rate species). New information is also presented from the flow cytometry studies in which it was found that the same cell population had similar (and substantial) levels of receptors for both testosterone and estrogen. This conclusion can be drawn because the T-BSA-FITC and E-BSA-FITC had the same amount of fluorescence and hormone covalently linked to the BSA, and the same number of cells (10,000) were counted for each steroid.

The initial experiments utilized water-soluble testosterone, water-soluble 17β-estradiol, and β-cyclodextrin in vehicle (Hank's buffer) as the control. This control was selected because the steroids were made water-soluble through encapsulation in β-cyclodextrin. Because the average level of increase observed in



controls is very small compared to the average increase observed for either hormone, it can be concluded that both testosterone and 17β-estradiol induce a rapid increase in intracellular calcium ions in osteoblasts. The rapidity of the changes in $[Ca^{2+}]_i$ is a hallmark of signaling through a plasma membrane receptor. The small responses observed in controls may reflect stimulation of mechanoreceptors by fluid flow over cell surfaces, as shown in osteoblasts by Duncan et al. [1998].

To characterize the $[Ca^{2+}]_i$ response induced by testosterone and 17β-estradiol, the calcium binding agent EGTA and the voltage-gated calcium channel blocker verapamil were used to identify the origin of increased ionic calcium in the cytosol ($[Ca^{2+}]_i$) as being from intracellular or extracellular sources. After EGTA treatment, testosterone and 17β-estradiol both showed only moderate increases compared to treatments before EGTA treatment, by a factor of about two-thirds. This indicates that a substantial portion of the increased $[Ca^{2+}]_i$ is from external sources. The remaining portion of the increase elicited by either hormone must come from intracellular stores. Furthermore, before treatment with EGTA, the hormones both cause a steady influx in Ca^{2+} after peaking, which appears as a plateau in the spectrofluorimeter-derived curves. This plateau effect is not seen by hormone treatment following treatment with EGTA; rather, the levels quickly rise, peak, and then decline. This steady influx of elevated $[Ca]_i$ is indicative of a sustained extracellular Ca^{2+} response and not merely a release from intracellular stores.

Verapamil also partially blocked responses to testosterone and 17β-estradiol. When cells were treated with both verapamil and EGTA, neither hormone elicited a response. The increase in $[Ca^{2+}]_i$ in the presence of verapamil may represent a release of Ca^{2+} from intracellular stores, because verapamil blocks voltage-gated calcium entry channels. In the presence of EGTA, those intracellular stores could not be

Fig. 3. Flow cytometry characterization of the fluorescence intensity of 10,000 osteoblasts exposed to testosterone (T) or estradiol (E) conjugated to bovine serum albumin (BSA; 10^{-6}M). Graphs are presented as log of FITC versus number of cells (10,000 total) for **A:** no treatment, **B:** BSA-FITC, **C:** T-BSA-FITC, **D:** E-BSA-FITC. Results are representative of three replicates from four separate cell isolations.

refilled, and so with both verapamil and EGTA present, only a very small or no increase in $[Ca^{2+}]_i$ would be possible. Accordingly, it appears that testosterone and 17β-estradiol cause an increase in $[Ca^{2+}]_i$ through both releasing intracellular stores and opening voltage-gated calcium channels.

After characterizing the intracellular calcium response due to testosterone and 17β-estradiol, the hormones conjugated to BSA (T-BSA and E-BSA) were used to show that the observed increase in $[Ca^{2+}]_i$ was due, at least in part, to specific binding to the plasma membrane hormone receptors, because the covalently attached protein (BSA) prevented entry of the hormone into the cell. The occurrence of a second-messenger response, namely a rise in intracellular Ca^{2+}, along with the rapidity of the response, indicates the presence of a plasma membrane receptor. Both T-BSA and E-BSA treatment resulted in increases similar to those observed when unconjugated, free hormones were administered (Table I).

Flow cytometry was used to determine the amount of fluorescence on a cell-by-cell basis following exposure to T-BSA-FITC and E-BSA-FITC along with appropriate controls. The T-BSA-FITC and E-BSA-FITC treated cells were approximately four times more fluorescent than cells treated with BSA-FITC control. Plasma membrane receptors were inferred because the covalent linkage to BSA prevented internalization. Because virtually all 10,000 cells counted and assessed for each experiment are labeled in each of these two treatments, and because the population of cells used in each treatment is the same (aliquots of the same isolation), it is concluded that osteoblasts simultaneously possess plasma membrane receptors for both testosterone and 17β-estradiol. The area of the peaks, an indication of fluorescence yield, for both T-BSA-FITC and E-BSA-FITC were nearly identical; this is an indication that receptor concentration per cell was similar for both hormones.

The present study focused on osteoblasts obtained from male chickens and revealed that specific binding sites for both testosterone and 17β-estradiol are present on the same cells. It is likely that osteoblasts derived from female chickens would likewise have plasma membrane receptors for both hormones. However, this needs to be shown experimentally. The possibility that one receptor binds both hormones hasn't been ruled out, although based on what is known about nuclear receptors, this would be a surprising result. It is interesting that Abu et al. [1997] reported the pattern and number of osteoblasts expressing the classical nuclear androgen receptor in human bone were similar in both males and females. Increased $[Ca^{2+}]_i$ in response to testosterone supports the findings of Lieberherr and Grosse [1994]. A calcium-signaling response to estrogen has been reported by Massas et al. [1998]. Lieberherr et al. [1993] also reported a calcium response to estrogen, but in the latter case the response was restricted to osteoblasts derived from female rats.

As a whole, the data in the present study indicate that cultured, primary osteoblasts from males possess substantial amounts of plasma membrane receptors for both testosterone and 17β-estradiol. Furthermore, ligand occupancy of these receptors causes a sudden intracellular calcium increase by releasing intracellular calcium stores and by opening voltage-gated calcium channels. Also, it appears that influx through voltage-gated calcium channels causes the intracellular calcium level to plateau, i.e., remain high for as much as 200 s, as shown by the calcium-channel blocking experiments, a phenomenon reported by others [Lieberherr et al., 1993; Lieberherr and Grosse, 1994].

The data suggest that the plasma membrane hormone receptors occur in a manner that is independent of gender. Although the traditional nuclear hormone receptors will continue to be of central importance in understanding long-term bone metabolism, further study of the plasma membrane receptors will likely prove to be an important piece of the puzzle, especially in understanding short-term regulation of bone metabolism wherein cross-talk among signaling pathways is likely to occur. With the presence of the plasma membrane sex steroid receptors in osteoblasts being shown by several investigators, additional characterization of the receptors and how they may function in both males and females is warranted. Isolation of these receptors and the identification of the genes that encode them is necessary to gain better understanding of how these receptors function in vivo.

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